Approach for production of sensitive to oxidation and aggregating proteins in *E. coli* at the example of a heterologous ribonuclease inhibitor

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zur Erlangung des akademischen Grades

Doktor der Ingenieurwissenschaften
- Dr.-Ing. –

genehmigte Dissertation

Promotionsausschuss:

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Tag der wissenschaftlichen Aussprache: 02.07.2012

Berlin 2013
D83

Zusammenfassung

Die Mitglieder der Gruppe der Angiogenin/Ribonucklease-Inhibitoren (RIs) sind in heterologen Wirten schwierig zu exprimieren.

In der vorliegenden Arbeit werden verschiedene Strategien beschrieben, mit denen RI mittels der Fed-batch Fermentation produziert werden können. Diese verschiedenen Strategien resultieren in hohen Produktionausbeuten, (a) als lösliches Fusionsprotein mit Maltosebindungsprotein (MBP), oder als korrekt prozessiertes Protein (b) im Periplasma, bzw. (c) im Zytoplasma.

Für das Scale-up aller Prozesse wurde die Fed-batch Technologie in Form der enzymbasierten Substratfreisetzung (EnBase®) angewandt, die es erlaubt, den Wachstumsmodus im Hochdurchsatzscreening und im Schüttelkolben, ähnlich wie im finalen Bioreaktor zu kontrollieren.

Die angewendeten Kultivierungsstrategien erlauben unter Fed-batch Bedingungen eine schnelle und systematische Evaluierung der Faktoren, die die Faltung von RI beeinflussen, wie z.B. die Nutzung reduzierender Medienzusätze und/oder die Koexpression von molekularen Chaperonen. Auf der Basis dieser Daten konnten dann ohne weitere Optimierung die direkten Hochzelldichte-Fed-batch-Prozesse im Bioreaktor etabliert werden.

Es konnte weiterhin gezeigt werden, dass die hier neu entwickelte Strategie zur Herstellung authentischen RIs, basierend auf der Modulation des Redox-Niveaus durch Zugabe von Dethiothreitol, auch für die Produktion anderer leicht aggregierender prokaryotischer und eukaryotischer Modellproteine vorteilhaft ist.

Die aktuelle Studie dokumentiert, wie detailliertes physiologisches Wissen in Kombination mit Hochdurchsatz-Screening-Strategien erfolgreich für die systematische Entwicklung von Bioprozessen für Proteine mit kompliziertem Faltungsverhalten genutzt werden kann.

Abstract

The group members of angiogenin/ribonuclease inhibitors (RI's) are difficult to produce proteins in heterologous hosts.

Here we describe several RI fed-batch production process development and scale-up strategies, which resulted in high production levels of soluble RI as a cytoplasmic fusion protein with MBP, as a periplasmic correctly processed authentic RI, and an authentic RI in the cell cytoplasm.

For scale-up of all processes the fed-batch cultivation technology was applied in the form of the EnBase® biocatalysis-based feeding system, allowing from the early screening steps to the final bioreactor process to maintain highly similar growth mode. The utilized cultivation strategies enabled a quick and systematic evaluation of the original RI folding approaches based on the utilization of reducing medium additives and/or the co-expression of molecular chaperones at the small scale fed-batch production mode. The obtained data allowed to develop an efficient RI production process at the high cell density in stirred-tank bioreactor.

The newly developed RI folding approach, based on the cytoplasmic redox modulation by medium supplementation with dithiothreitol (DTT), was applied and verified for the production of several aggregate-prone eukaryotic and prokaryotic model proteins.

The actual study shows how the knowledge in cell physiology combined with the high-throughput screening strategies can be successfully applied for a straightforward bioprocess development with the aim to produce proteins requiring sophisticated folding.

The keywords: Ribonuclease inhibitor, *Escherichia coli,* optimization of expression, redox conditions, redox sate, fed-batch, Enbase

Acknowledgements

Mainly, this work was carried out in R&D centre of Fermentas UAB Lithuania, Vilnius, which now is a part of Thermofisher scientific. The significant part of the experimental data was derived in the University of Oulu (laboratory of Bioprocess Engineering, Finland), Technical university of Berlin (Bioprocess engineering laboratory, Germany) and in Institute of Pharmaceutical Biotechnology/Institute for Microbiology (Greifswald, Germany).

Firstly, I would like to express my deepest gratitude to my former supervisor Prof. Arvydas Janulaitis, who help me to initiate this research project, encouraged me to pursuit and realize my ideas and who always enthusiastically supported me.

I would like to express deep gratefulness to my teacher Prof. Peter Neubauer, who supervised me during this project. His very profound, knowledge, expertise, excellent guidance and enthusiasm allowed me to learn and discover new things. I will never forget those interesting brain-storming-discussion, which were driving this research project ahead. I am very grateful for opportunity to spend significant amounts of my research time in his laboratories.

Also I would like to thank the “Fermentas UAB” team, who always were helping me to solve various technical issues and for the honest support.

I would like to thank to Prof. Thomas Schweder and his team for opportunity to perform the proteomic studies in the Institute for Microbiology, University of Greifswald, and for helping me during my research stay.

Special thanks to Kathleen Szeker (Bioprocess Engineering laboratory, TU Berlin), for helping me to collect the required documents, and help during the thesis submission.

Thanks to BioSilta and its team for opportunity to use Enbase® cultivation technology during my research.

Finally, I would like to express my deepest thankfulness to my family members and my friends, who always believed in me, encourage me, supported me and were helping me.

This work is dedicated to my family...

List of original articles

The presented PhD theses are based on the following papers:

Paper I:

Šiurkus J, Panula-Perälä J, Horn U, Kraft M, Rimseliene R, Neubauer (2010) Novel approach of high cell density recombinant bioprocess development: optimisation and scale-up from microliter to pilot scales while maintaining the fed-batch cultivation mode of E. coli cultures. Microb Cell Fact 9:35.

Paper II:

Šiurkus J, Neubauer P (2011) Heterologous production of active ribonuclease inhibitor in Escherichia coli by redox state control and chaperonin co-expression. Microb Cell Fact 10:65.

Paper III:

Šiurkus J, Neubauer P (2011) Reducing conditions are the key for efficient production of active ribonuclease inhibitor in Escherichia coli. Microb Cell Fact 10:31.

Paper IV:

Šiurkus J, Schweder T, Neubauer P (2012) Proteome profiles for cytoplasmic production of RNase inhibitor in *Escherichia coli* with DTT mediated folding in shake flask and bioreactor cultures. Manuscript.

Other publications:

Juozas Šiurkus, Peter Neubauer UK Patent Application No. 1101794.4, Production of Proteins

The scientific results were presented in the following international conferences:

Šiurkus J, Neubauer P. 2008. Novel Approach of High Cell Density Recombinant Bioprocess Development, Optimization and Scale-up from micro – to final scales, with maintenance of fed-batch cultivation mode of E. coli Cultures. Oral presentation (PN) at the Recombinant protein production conference RPP2008, Porto Conte Bay, Sardinia, Italy, 24-28 8 2008.

Šiurkus J, Neubauer P. Process development for oxidation sensitive proteins – the Ribonuclease Inhibitor case. Oral presentation (JS) at RPP 6 - A comparative view on host physiology, Vienna, Austria, 16 – 19 2 2011.

Contents

1 Introduction

In this work we focussed on heterologous expression of oxidation and aggregation-prone proteins in *E coli* based constructs. Therefore, as a model protein we selected the eukaryotic ribonuclease inhibitor (RI), which is a member of conservative ribonuclease inhibitor protein group. A model protein used RI (~49 kDa) in this work shows 79-82 % homology to the other well characterized RNase ribonuclease/angiogenin inhibitors: from human (hRI), rat (rRI), mouse (mRI) and porcine (pRI). The eukaryotic angiogenin ribonuclease inhibitors are distinguished by an unusual native structure – a hydrophobic horse shoe like backbone comprising of 18 % of leucine residues and 30-32 reduced cysteins, i.e. approx 7 % of total amino acid pool.

Literature documented ribonuclease inhibitor production attempts showed that it is very complicated to efficiently propagate the members from this protein group as heterologous proteins due to their tendency to accumulate in the insoluble protein fraction. Therefore, the challenge in this work was to identify the key genetic, cultivation, induction and synthesis factors limiting RI propagation in the native and soluble state in *E. coli*.

The presented work comprises several tightly related steps, which mainly focuses on the issues related to an efficient RI propagation in *E. coli* cells.

In the first part of the work the accumulation of soluble RI fused with various N-terminal tags was systematically investigated under the substrate-limited fed-batch cultivation mode, using the constructs harbouring different promoters and ribosome binding sites. The novel “pump-independent” fed-batch cultivation technique EnBase® allowed to evaluate these constructs in the microwell plates and shaking flasks at the substrate-limited growth conditions, which are highly similar to the fed-batch cultivation conditions in the stirred-tank bioreactor.

The same fed-batch screening approach was utilized in the second part of the work to identify the constructs, which are suitable for the

efficient RI expression in bacterial periplasm. However, the RI periplasmic propagation strategy did not produce the desired results, thus, the following trails were mainly designed by considering the key features of RI, i.e. hydrophobicity and sensitivity to oxidation. The proper folding and accumulation of soluble RI was observed in the constructs harbouring weak expression elements after modification of the redox environment with low molecular weight reducing agents and production at low production temperature. The same procedure and production construct were successfully applied also for the production of properly folded RI in cytoplasm. The expertise and data obtained in the first part of the work served for scale-up and transfer of the periplasmic and cytoplasmic RI small scale fed-batch processes with the redox modification to the stirred-tank bioreactor.

In the third part of the work the RI folding possibilities using the T7 RNA polymerase based system in *E. coli* were investigated. The RI synthesis trails based on the co-expression of the main *E. coli* chaperons – DnaK/J/GrpE and GroEL/ES, and medium supplementation with the reducing agents revealed, that besides the redox state the high RI aggregation level or improper folding is caused by the bottlenecks in protein folding machinery at the late stages of the cellular protein folding pathway. The knowledge obtained during the first and the second parts of the work served in developing of previously not documented recombinant fed-batch production process, where the folding of the target protein at the high cell densities in stirred-tank bioreactor was facilitated by the chaperon co-expression and cellular redox modification.

The versatility of the established recombinant synthesis approach was applied for the production of two eukaryotic and two prokaryotic origin aggregation–prone model proteins in various *E. coli* based expression systems. In all the cases, a medium supplementation with reducing agents during the recombinant synthesis course either facilitated the model protein accumulation in the soluble fraction, and/or increased protein's activity per biomass.

Finally, in the last part of the work the comparative proteome analysis was conducted using the construct of RI expression in

cytoplasm with aim to elucidate redox and stress responsive proteins. After the RI propagation in the shaking flasks and stirred-tank bioreactor the changes in the host proteome were revealed in the levels of more than 50 proteins, which were acting against the multiple stresses, i.e. induced in response to the redox modification, low aeration and low recombinant production temperature.

2 Review of literature

The prokaryotic/bacterial protein production platforms are attractive due to the rapid cell mass accumulation on cheap substrates, profound physiological and genetical characterization of the host organism, easy genetical manipulations and the availability of large number of the expression vectors and strains.

2.1 *E. coli*

Among the prokaryotes, gram-negative bacterium *Escherichia coli* is still the most extensively utilized for recombinant protein production. The genetic engineering techniques allowed to accommodate *E. coli* strains for recombinant protein propagation. The modifications of *E. coli* strains were performed to overcome low productivity, product proteolytic degradation, inability to form disulfide bridges and/or to reduce product aggregation (Makino et al. 2011). For example, the inactivation of Lon and OmpT proteases increased heterologous protein stability (Makino et al. 2011), or inactivation of *trx* and *gor* genes resulted in the improvement of disulfide bond formation in the *E. coli* cytoplasmic space (Bessette et al. 1999).

The expression of rare-codon-possessing genes was improved by the co-expression of eukaryotic tRNA genes from additionally introduced plasmids (Jonasson et al. 2002). Finally, special derivatives C41 and C43 were designed for the propagation of toxic membrane-associated proteins, such like – subunit of F_1F_o ATP synthase (Freigassner et al. 2009).

The recombinant gene production in *E. coli* constructs was enabled by using T7 RNA polymerase expression systems and several other popular expression systems. The most extensively used are the synthetic *tac and trc* promoters, the λ-phage *pL/pR* promoters, the tetracycline inducible *tetA* promoter, the L-arabinose inducible *PBAD*

promoter, and the L–rhamnose inducible *rhaPBAD* promoter (see sections bellow and Samuelson et al. 2011).

In *E. coli* platforms, besides the cytoplasmic space, the recombinant protein accumulation could be directed to the periplasmic space or even exported to the cultivation medium (Jong et al. 2010, Su et al. 2012).

Despite the outstanding characteristics of *E. coli* host, due to its limited folding and post-translation modification capabilities, often the heterologous proteins are forming cytoplasmic and/or periplasmic aggregates, and/or are incapable to obtain their native structure and full biological activity (Baynex et al. 2004). In addition, the accumulation of lipopolysaccharides/endotoxins is a huge disadvantage in pharmaceutical protein production. The extended purification schemes are required in order to liberate proteins from endotoxins (Terpe et al. 2006).

2.2 The factors influencing recombinant protein production in *E. coli*

In this part the main aspects related to heterologous protein production and accumulation in *E. coli* strains will be briefly reviewed.

2.2.1 The *E. coli* promoter expression systems

The promoter is an upstream element, controlling the initiation and elongation rate of the mRNA synthesis. The promoter is composed of hexameric sequences 10 and 35 base-pairs upstream from the gene coding region and the space between them (Harley et al. 1987, Lisser et al 1993).

Depending on the promoter regulating elements, the initiation of recombinant protein synthesis, so called induction, could be performed by the addition of various chemicals, temperature shifts and creation of different stress and/or starvation conditions (Makrides et al. 1996). The difference between the transcription rate under restricted conditions and the synthesis rate after induction is defined as an

induction range. Higher induction range is preferred, because the tightly controlled promoters, which are stipulating very low basal expression level, efficiently divide the recombinant production process in to two phases - biomass accumulation and recombinant synthesis.

2.2.1.2 T7 RNA polymerase promoter system

In *E. coli* constructs, harbouring T7 RNA polymerase promoter system the propagation of the recombinant gene mRNA is driven by the T7 phage RNA polymerase, which is 5 times faster than the RNA polymerase of *E. coli* (Studier et al. 1990). The T7 RNA polymerase is supplied in trans, by the IPTG inducible *lacUV/lacO* promoter/operator system in the T7 RNA polymerase expression cassette (known as "DE3"), which is integrated into the chromosome of *E. coli* host via the λ- phage mediated recombination (Studier et al. 1986). The T7 RNA polymerase expression system is capable to achieve the level of recombinant protein accumulation of 50 % of the total cellular proteins (Studier et al. 1986 and 1990). However, a very high recombinant gene expression level from the strong *T7* promoter may lead to the ribosome destruction (Dong et al. 1995) and/or target protein inability to encounter the native conformation and accumulate in insoluble aggregates (Jürgen et al. 2010).

The target gene synthesis induction level favourable for the accumulation of soluble protein could be adjusted by the IPTG titration. However, the precise recombinant expression control is possible only in T7 DE3 strains, which are deficient in lactose permease gene *lacY* (Picaud et al. 2007).

The T7 RNA polymerase expression systems are considered as "leaky", thus the expression of very toxic proteins could be hampered. However, the expression leakage could be reduced by the additionally inserted *lac* operator *lacO* sequence downstream the *T7* promoter, and/or by the co-expression of T7 RNA polymerase inhibiting T7 lysozyme (Studier et al. 1990).

2.2.1.3 *Tac* and *Trc* promoter system

The *trc* and *tac* promoters are derived from the naturally occurring *trp* and the modified *lac* promoter l*acUV5*. The “-35” region is taken from the *trp* promoter and “-10” region from *lacUV* promoter (De Boer et al. 1983).The main difference between *trc* and *tac* promoters is the distance between “-35” and “-10” regions, corresponding to 16 bp in *tac* and 17 bp in *trc* (Brosius et al. 1985). The *tac* and *trc* promoters are allowing to reach the recombinant protein accumulation up to 15 – 30% of the total cellular proteins (Brosius et al. 1985). Compared to the T7 RNA promoter system, the *tac*/*trc* promoter system is much leakier, thus the synthesis of toxic proteins in the constructs harbouring these promoters is even more problematic (Otto et al. 1995). The basal expression level of the recombinant gene in *tac/trc* system could be reduced by increasing the LacI repressor synthesis level in the cell. For these proposes the *lacI* or *lacIQ* alleles could be cloned in the same expression vector or provided in trans from an allele integrated in the host genome, or from a separate plasmid (Glascock et al. 1998).

2.2.1.4 *araBAD* promoter system

Contrary to the previously discussed IPTG inducible promoters, the L-arabinose dependent promoter *araBAD* drives very low basal transcription (Lee et al. 1987), which could be even further diminished by supplementing the cultivation medium with glucose (Miyada et al. 1984) and/or constitutive co-expression of the *araBAD* repressor AraC (Khlebnikov et al. 2002). The expression level of *araBAD* promoter is linearly depended on the inducer concentration, thus the performance of *araBAD* promoter could be tightly regulated by the external arabinose concentration without any special genetical modification of the expression host (Guzman LMet al. 1995). The *araBAD* promoter is considered weaker than the previously described *trc* and *tac* promoters. The efficient expression control of *araBAD* promoter could be even combined with the performance of strong *T7* promoter. For example, in the “BL21–AI” strain (Invitrogen) the expression cassette

harbouring T7 polymerase gene under araBAD promote is integrated into the host genome (see Invitrogen web page).

2.2.1.5 *tetA* promoter system

The tetracycline (Tc) or anhydrotetracycline (aTc) inducible *tetA* promoter/operator expression systems have been successfully applied and are currently widely used for the synthesis of recombinant proteins in *E. coli* cells (Skerra et al. 1994). The regulatory elements of *tetA* promoter/operator system are taken from the *Tn10* Tc^R gene, which naturally is encoding for TetA(B) proteins, the members of the so called “tetracycline efflux pump” (Hillen et al. 1994). Naturally the tetracycline efflux pump removes tetracycline from the cell (Chopra et al. 2001). The *tetA* promoter is repressed by TetR repressor, which dissociates from the *tet* operator due to conformational changes in response to the presence of tetracycline (Meier et al. 1988). The *tetA* promoter/operator expression systems are considered as very tightly controlled, and, contrary to the *lac* operone based expression systems are, not functionally coupled to the host physiological regulation (like cAMP level depended catabolic repression) (Bertram et al. 2008). The performance of the *tetA* promoter is depended on the inducer concentration in the cultivation medium and/or cell density. In some cases, in order to maintain *tetA* promoter fully induced during the whole production course, the repeated addition of aTc is required (Neubauer et al. 2007)

2.2.1.6 Phage λ *pL* and/or *pR* promoter system

The thermo inducible expression system, based on the λ-phage gene expression controlling promoters *pL* and/or *pR* and their thermolable mutant repressor *cI857,* became widely used for recombinant gene expression in *E. coli* hosts (Valdez-Cruz et al. 2010). The *λPL/Pr-cI857* system is attractive because expensive and/or toxic chemical inducers could be avoided.

The induction of *λPL/Pr-cI857* expression system and recombinant synthesis cannot be carried out at the temperature bellow 37 ℃,

meaning that the accumulation of highly aggregating proteins in soluble form could be very problematic (Vallejo et al. 2002).

The pL (major leftward) or pR (major rightward) promoters are considered as strong promoters and are capable to facilitate the target protein accumulation up to 30% of the total cellular proteins (Remaut et al. 1981). The gene encoding the thermo-lable bacteriophage λ repressor mutant CI857 is positioned in the same expression vector, with the aim to ensure a complete repression of *pL* or *pR* promoters. Even a single copy of *cI875* gene generates the amount of repressor, sufficient to ensure the complete inhibition of *pL* and/or *pR* promoters, even if they are present in the high-copy plasmid (Remaut et al. 1981).

Prior to synthesis induction the constructs harbouring *λPL/Pr-cI857* expression vector are cultivated at the temperatures of 28-32 ℃, at which the λcI857 repressor is functionally stable, and, by interacting with three operator domains (oL3/oR3, oL2/oR2, oL1/oR1), is preventing the transcription of the target gene (Dodd et al. 2001). The up-shift of cultivation temperature from 28-32 ℃ to 40-42 ℃ stipulates the release of λcI857 repressor from the operator domains and permits the transcription of the target gene. In order to prevent the re-coupling of repressor with the operator, the temperature is kept above 30 ℃ during all recombinant synthesis course (Tabandeh et al. 2004).

2.2.1.7*cspA* promoter system

The *cspA* promoter system allows to perform recombinant protein expression at the low synthesis temperature (10 - 25 ℃) (Vasina et al. 1996), which is favourable for the propagation of toxic, proteolytically-sensitive and aggregation-prone proteins (Mujacic et al. 1999, Qing et al. 2004). The *cspA* promoter is taken from the *E. coli* gene encoding for CspA the major cold shock protein, the cellular production of which highly increases (more than tenfold) in response to temperature down shift from 37 ℃ to 15℃ (Yamanaka et al. 1999). In the nature, at the low temperatures the CspA activates the transcription of “cold-shock” responsive genes. In addition, CspA refolds bulk mRNA, which due to

the low temperatures is imbedded in the secondary structures (Gualerzi et al. 2003).

The CspA synthesis is temperature dependent due to the 159 nt-long 5' – untranslated region (5'-UTR) at the 5' end of the *cspA* gene transcripts. The 5'-UTR of *cpsA* mRNA is unstable at 37 ℃ and highly stabilized at temperature bellow 20 ℃ (Jiang et al . 1996). Therefore, the *cspA*-promoter-based expression vectors unconditionally contain the downstream positioned 5'-UTR region. The expression leakage in cspA systems could be reduced by the addition of *lac operator* (for example pCold™ from Takara Inc.). The de-repression of *cspA* promoter can occur 1-2 hours after the temperature downshift due to the host physiological acclimatization to the low temperature conditions (Vasina et al. 1998). In order to extend the performance of *cspA* promoter the inactivation of host gene *rbfA,* encoding the30S ribosome associated protein RbfA, is required (Vasina et al. 1998). The RbfA the cold sensitive, non functional ribosomes turns into functional and cold-adapted (Xia et al. 2003).

2.2.2 Recombinant gene translation

2.2.2.1 Factors essential for efficient gene translation

Gene translation is the key expression step, which determines the end result - the peptide accumulation level. The translation efficiency is depended on the translation initiation, which is mainly related to the structural features of mRNA at the 5' end (Sørensen et al. 2005, Laursen et al. 2005).

The Shine-Dalgarno (SD), sometimes called the ribosome-binding sequence (RBS), is the untranslated mRNA region located upstream of the coding sequence and interacting with the complementary 3' end of the 16S ribosomal RNA (rRNA) during the initiation of translation (Shine et al. 1974). The efficiency of the translation initiation and/or translation is depended on the nucleotide composition of the SD sequence and the regions located downstream and upstream from it (Warburton et al. 1983, Ringquist et al. 1992, Jin et al. 2006 and

Nishizawa et al. 2010). In addition, the distance between the SD and the start codon may also have a huge impact on the gene translation efficiency (Chen et al. 1994, Paik et al. 2006).

In *E. coli* cells the translation could be initiated from several start codons. The AUG start codon is the most efficient and most frequently used–at the frequency of 83%, compared to the GUG and UUG with frequencies of 14% and 3%, respectively (Ringquist et al. 1992, Ma et al. 2002).

The translation is terminated when the translating ribosome “meets” one of the stop codons: UAG, UGA or UAA (Tuite et al. 1994). The release of ribosome from the mRNA is mediated by three release factors: decoding - RF1, RF2, and stimulatory - RF3 (Tuite et al. 1994, Tate et al. 1996). The RF1 release factor terminates the translation at UAG, and the RF2 terminates the translation at UGA and UAA codons (Rydén et al. 1984, Craigen et al. 1987).

The translation termination efficiency is also depended on the first single nucleotide after the termination codon (Tate WP et al. 1995, 1996). For example, the most efficient translation termination sequence in *E. coli* is the UAAU (Poole et al. 1995).

The formation of secondary structures in the 5’ end of the mRNA results in the termination of the translation initiation due to restricted accessibility for the 30S ribosomal subunit to the SD sequence and/or initiation codon (de Smit et al. 1990, Seo et al. 2009). The formation of secondary structures in the 5’ end of mRNA could be minimized by modifying the SD sequence and the region between the SD and AUG (Paik SY et al. 2006), by carrying the recombinant production at the higher temperatures (Szeker K et al. 2011), by enriching the SD sequence with the adenine and thymidine residues, and, finally, by utilizing the translationally coupled systems (Makrides SC et al. 1996).

The translation efficiency could be enhanced by using the special elements positioned upstream of the SD. For example, the translation of the lacZ gene was 110-fold enhanced due to the introduction of the 9-base sequence from the T7 phage gene 10 (g10-L) in the position upstream of the SD sequence (Olins et al. 1989). The same SD-upstream element increased the accumulation level of the difficult to

express proteins from human placenta - PP9 and PP15, in the *E. coli tac* promoter harbouring expression system (Lehmeier et al. 1992).

The U-rich elements positioned upstream of the SD also act as the translation enhancers. The 30-base long un-translated region taken from the *E. coli atpE* gene (McCarthy et al. 1985) resulted in significant synthesis improvement of the human interleukin-2 and interferon-β (McCarthy et al. 1986).

Finally, the special elements positioned downstream of the start codon also impact the translation efficiency and, thus, the recombinant production. For example, the downstream box (DB) sequences from the T7 bacteriophage genes 0.3 and 10 located at the positions +15 - +26 and +9 - +21 are well described translation enhancers (Sprengart et al. 1990 and 1996, Rush et al. 2005). The non – modified DB element significantly enhanced the accumulation levels of the staphylococcal nuclease, chicken muscle adenylate kinase, human TF-1 cell apoptosis related protein TFAR19, and the hypothetical protein TTE0085 from *T. tengcongensis* (Zhang et al. 2003).

2.2.2.2 The impact of mRNA composition on translation efficiency

The translation efficiency of recombinant gene is depended not just on the previously discussed initiation factors, but also on the nucleotide composition of the gene, which determines the codon usage during the recombinant gene translation (Lithwick et al. 2003). The pool of tRNAs in the cell is correlating to the codon preference and “prevalent” gene expression levels (Ikemura et al. 1981 and 1985). The major pool of tRNAs in the cell is used for the translation of highly expressed genes and the minor - for the translation of low expressed genes (Gouy et al. 1982, Kane et al. 1995, Karlin et al. 1998 and 2001). The usage frequency of synonymous codons varies significantly between the prokaryotic and eukaryotic organisms (Moriyama et al. 1998). Therefore, the expression of heterologous genes of eukaryotic origin in bacteria cells is quite limited. Especially when the target gene is enriched in the codons preferred low and

used rarely in the production host (Gustafsson et al. 2004). In the *E. coli* cells the most problematic are the codons of the lowest frequency: AGG/AGA, CGA – coding for arginine, CUA- leucine, AUA- isoleucine, and CCC - proline (Kane et al. 1995).

The expression of the heterologous gene possessing rare for the host codons (i) reduces the recombinant gene translation rate, causes (ii) mistranslation (Calderone et al. 1996, McNulty et al. 2003), premature translation termination and (iii) frameshifts (Farabaugh et al. 2000). In addition, the rare codons are slowing the ribosome trafficking and, thus, create open regions in the mRNA, which makes the recombinant mRNA prone to degradation by RNases (Deana et al. 1996, Goetz et al. 2005). Finally, the high abundance of rare codons in the heterologous gene could alter the synthesis of cellular proteins and, thus, result in the cell growth inhibition or even death (Zahn et al. 1996, Olivares-Trejo et al. 2003). This phenomena is caused by the depletion of cellular rare tRNAs (for example $tRNA^{AGA/AGG}$), when the ribosomes are stalling at the rare-codon positions (for example AGA/AGG) (Zahn K et al. 1996, Olivares-Trejo et al. 2003, Jacinto-Loeza et al. 2008).

One of the common strategies, allowing to at least partially overcome the negative rare-codon effects in the recombinant synthesis in *E. coli* cells, is based on the co-expression of the rare tRNAs, for example, genes for the $tRNA^{AGA/AGG}$(Arg) (Brinkmann et al. 1989) and $tRNA^{AUA}$ (Ile) (Del Tito et al. 1995). However, the artificially increased levels of rare tRNAs might provoke the target protein aggregation due to the highly increased and/or imbalanced target protein translation (Rosano et al. 2009).

A novel codon optimization approach is based on the rational gene design (Villalobos et al. 2006, Welch et al. 2009) or “codon harmonization” between the codon-usage frequencies in the expression host and the codon composition of the recombinant gene (Angov et al. 2011).

Recently, the Gvritishvili and co workers compared the rational gene design approach versus the co-expression of the rare tRNAs and showed that the accumulation of a thioredoxin (Trx) fusion with the

pigment epithelium-derived factor (PEDF) was highly improved after the rational recombinant gene design (Gvritishvili et al. 2010).

2.2.3 Protein folding in *E. coli* cytoplasmic space

In the prokaryotes the proper nascent peptide isomerization, stabilization, folding, refolding, dissolving of the aggregated proteins and disposal of the damaged proteins is carried out by chaperons and proteases - proteins belonging to the group of so called “Heat-shock response proteins” (Hsp’s). The synthesis levels of more than 30 Hsp’s are tightly controlled by the Heat-shock sigma factor RpoH (δ^{32}) (Zhao et al. 2005). The stability and synthesis level of RpoH in the cell depends on the temperature shifts and other stresses, including the accumulation of misfolded/damaged proteins during the recombinant production (Hoffmann et al. 2004, Valdez-Cruz et al. 2011).

The major protein folding modulators – chaperons, are sorted according to their molecular weight/size into several groups: small Hsp’s – IbpA/IbpB (15 kDa) (Laskowska et al. 1996), Hsp31 (DJ-1 superfamily) (Sastry et al. 2002), Hsp33 (Vijayalakshmi et al. 2001), GroEL (Hsp60) with cofactor GroES (Hsp10) (Kusukawa et al. 1989), DnaK (Hsp70) with cofactors DnaJ (Hsp40) and GrpE (Bukau et al. 1998), HptG (Hsp 90) (Bardwell et al. 1987, Wandinger et al. 2008) and ClpB (Hsp 100) (Mogk et al. 2003). In addition, based on the action mechanism (functionality) the molecular chaperons are subdivided into folding (GroEL), holding (DnaK), disaggregating/refolding (ClpB, IbpA/B), and independent from the RpoH peptidyl-prolyl cis/trans isomerases – PPIases (TF) (Arsène et al. 2000). The folding/unfolding chaperons are requiring ATP for the maintenance of their competency to fold/unfold the client proteins (Gottesman et al. 2000).

The chaperons are acting at the different time points of the synthesized protein lifetime. After exiting the ribosome, the N-terminus of the de novo synthesized peptide is firstly bounded by the peptide stabilizing trigger factor (TF). The TF is associated with the ribosome via the ribosomal protein L23 in a ratio of 1:1. The TF is acting as a

chaperon – preventing the aggregation/folding of the client proteins, or as a peptidyl-prolyl cis/trans isomerase. The majority of the nascent proteins smaller than 20-30 kDa in molecular size are able to fold only with the aid of TF. The TF substrate binding specificity is overlapping with that of the chaperon DnaK (Deuerling et al. 2003, Hoffmann et al. 2010).

The larger nascent peptides are requiring the stabilization and, thus, are bounded by the DnaK/J/GrpE chaperon complex (Bukau et al. 1998). The DnaJ is a binding cofactor, which is mediating the DnaK chaperon interaction with the substrate's hydrophobic patches in the ATP-hydrolysis (by DnaK)-depended manner. After the ATP conversion to ADP the DnaK/J/GrpE protein complex turns into so called "high affinity ADP-bound state" (Suh et al. 1998, Perales et al. 2010). The release of the DnaK/J/GrpE complex from the client protein occurs after the GrpE mediated ADP conversion again to ATP (Harrison et al. 2003). The newly synthesized peptide might repeatedly interact with the DnaK/J/GrpE complex or could be transferred to the folding catalyzing GroEL/GroES chaperonin system (Bukau et al. 1998). The GroEL/ES is a barrel-shaped protein complex, possessing the cylinder walls, which are formed by two stacked GroEL homoheptameric rings (800 kDa). The cylinder is "closed" with a lid comprising 7 GroES co-chaperon subunits. The protein folding/refolding is ATP-depended and performed inside the cavity of the GroEL/ES barrel. The GroEL/ES folds 10-30 % of the newly synthesized peptides possessing the molecular sizes of 10-55 kDa (Masters et al. 2009, Azia et al. 2012).

The group of holding chaperons or holdases, like Hsp33, helps to prevent the protein aggregation by binding to the partially folded intermediates and participating in the folding of the oxidatively damaged proteins (Kumsta et al. 2009). In addition, holding chaperons are assisting the other chaperons in the refolding of the aggregated proteins (Mogk et al. 2003).

The disaggregating chaperon ClpB, acts in tandem with the small sHsp's IbpA/B and with the DnaKJ/GrpE chaperon group. After the solubilisation by ClpB, the client peptides are further processed by the

GroEL/ES until acquire native status (Doyle et al. 2009). Similar to ClpB, another chaperon HptG, in collaboration with DnaK, participates in the reactivation of the client proteins (Genest et al. 2011).

2.2.4 Recombinant protein folding in cytoplasmic space of *E. coli* by overproducing molecular chaperons

The cellular protein folding machinery plays a crucial role in the recombinant protein folding. Very often, the host folding machinery is not capable to facilitate the heterologous peptide folding. Thus, product is aggregating and/or not acquiring the native conformation. The co-production of the major molecular chaperons: TF, DnaK/J/GrpE and GroEL/ES, or their combinations (multiple chaperon co-expression) with a target protein is well known approach, allowing to improve the recombinant protein accumulation in a soluble state and/or increase its activity per biomass (Baneyx et al. 2003 and 2004, Makino et al.2011).

Interestingly, in some cases the “non-conventional” chaperons ClpB and IbpAB are capable to facilitate the target protein folding much more efficiently compared to the above mentioned “conventional” chaperon combinations (de Marco et al. 2007).

The reorganization/reprogramming of the cellular chaperon pathways is an alternative approach to the chaperon co-expression, allowing to improve the folding of the aggregation-prone recombinant proteins (Nannenga et al. 2011).

Worth mentioning that the co-expression of the certain chaperon groups doesn’t guarantee the increase of the target protein activity per biomass and/or its solubility (Yokoyama et al. 1998, Han et al. 1999, Zhang et al. 2002, Hu et al. 2007). In addition, in numerous times the overproduced chaperones were not just failing to facilitate the recombinant protein folding, but were decreasing the recombinant protein activities (Nishihara et al. 2000, Yoshimune et al. 2004) or even causing the cellular growth inhibition (Martínez-Alonso et al. 2010).

2.2.5 Fusion tags – folding and purification facilitators

2.2.5.1 N-terminus tags for target protein folding and recovery

The less sophisticated approaches, allowing to promote the protein folding, are based on the fusion of the target protein N terminus with the particular peptides or proteins, called fusion tags. The fusion tags could be also utilized as the affinity anchors for a faster target protein purification and/or detection (Terpe et al. 2003).

The hexahistidine (His_6) tag and an eight-amino acid peptide (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys), known as Strep-tag, are the most commonly used synthetic, low molecular weight tags (Schmidt et al. 1996 and 2007). The target protein tagged with the His_6 could be captured by a single-step chromatography from a crude cell extract by using the immobilized Ni^{2+} or Co^{2+} resin. In addition, the affinity of polyhistidine tag towards the metal ions could be enhanced by increasing the number of histidines in the tag (Khan et al. 2006). The specially engineered strains allow to reduce the levels of co-eluting proteins from the Ni^{2+} or Co^{2+} resins and, thus, to improve the purity of the target His-tagged proteins (Robichon et al. 2011).

The presence of the polyhistidine tag in the N or C terminus of the target protein might negatively affect the accumulation of the soluble recombinant protein (Woestenenk et al. 2004). Contrary to His_6 tag, in many cases the Strep-tag proved to be a better folding facilitator (Miladi et al. 2010).

A self-cleaving/aggregating tags is another interesting, recently presented group of the low molecular weight peptides, allowing to reduce the number of the purification-chromatography steps (Li et al. 2011). The induction of the assembly of the target proteins into active aggregates occurs *in vivo* due to the action of special peptides, for example, the amphipatic alpha peptide 18A and ELK16 (Xing et al. 2011). The self–cleaving affinity tag based recombinant synthesis approach was already successfully employed in the secretion and one-step recovery of the human antibody fragments from the periplasmic space of *E. coli* (Wu et al. 2011).

The maltose binding protein (MBP) from *E. coli* and glutathione S-transferase (GST) from *S. japonicum* are classical folding-aiding peptides, which, besides folding, also facilitate the fusion protein recovery from the protein extracts (Kapust et al. 1999, Lichty et al. 2005). The MBP-tagged proteins could be purified by using the resins with the immobilized sugars, amylase or dextrin (Riggs et al. 2001). The GST tag has an affinity to the reduced glutathione (Kapust et al. 1999).

The highly soluble cytoplasmic disulfide reductase thioredoxin (Trx) from *E. coli* is another prevalent folding-aiding tag (Terpe et al. 2003, Hire et al. 2009). Besides improving the fusion protein solubility, the N-terminal Trx tag also promotes the cytoplasmic formation of the correct disulfide bonds. However, this affect is possible only when the fusion protein is propagated in the cytoplasmic space of the *trxB* thioredoxin and/or glutathione reductase (*gor*) mutant (Stewart et al. 1998).

There are several other very efficient, but less prevalent folding facilitators: NusA (transcription terminator/anterminator) (Davis et al. 1999), Small ubiquitin-related modifier (SUMO) (Peroutka et al. 2011), malate dehydrogenase (Park et al. 2007), and the stress responsive protein Crr from the *E. coli* (Han et al. 2007).

Finally, the coupling of small synthetic affinity tags, for example, His_6 tag, with the larger folding-aiding tags combines the folding facilitation with a rapid recovery of the desired product (Bogomolovas et. al 2009).

2.2.5.2 Site specific proteases for N-terminus tag removal

The folding-aiding/purification tags might negatively affect the target protein activity and/or make the crystallisation difficult (Newby et al. 2009). The liberation of the target protein from the N-terminal tag is usually accomplished by the introduction of the specific target sequence, a linker between the target protein and the fusion tag, for the enzymatic cleavage with the site-specific or non-specific protease (Charlton et al. 2011). The fusion tag could be removed either *in vivo*

by tandem co-expression of the particular protease (Han et al. 1998, Nallamsetty et al. 2004,), or *in vitro* after the first purification step (Charlton et al. 2011).

There are several site-specific proteases commonly utilized for the removal of the fusion tags *in vitro*: TEV from the Tobacco Etch Virus (Sun et al. 2011), Hrv3C from the human rhino virus type 14 (Cordingley et al. 1989), Factor Xa (Jenny et al. 2003), catalytic subunit of the bovine enterokinase (Liew et al. 2005) and thrombin (Hefti et al. 2001). The selection of the protease system is usually accomplished empirically by systematic trails (Vergis et al. 2011, Gasparian et al. 2011). Very often, due to an inability to access the substrate (steric hindrance) and/or the protease inhibition by the buffer components the site-specific proteases are failing to cleave off the fusion tags (Shahravan et al. 2008).

2.2.6 Eukaryotic like protein modifications in *E. coli* cytoplasmic space

The disulfide-bond formation and protein isomeration in *E. coli* cytoplasmic space is limited due to the presence of reducing conditions, which are created by the thioredoxin and glutathione reductases (Ritz et al. 2001). Current approaches, aiming to facilitate the disulfide-bond isomeration, are based on the utilization of the prokaryotic or eukaryotic disulfide-bond formation and rearrangement catalyzing pathways**.** For example, the cytoplasmic co-expression of the *E*. *coli* periplasmic disulfide isomerase DsbC improved the folding of the Single-chain Fv (scFv) antibody (Kim et al. 2008), and the co-production of the sulfhydryl oxidase Erv1p from *S. cerevisiae* facilitated the folding of the alkaline phosphatase (PhoA) and phytase (AppA) (Hatahet et al. 2010). In addition, the same group showed that the disruption of the reducing pathways (*trxB* and *gshA*) is not essential for the PhoA and AppA folding, when the sulfhydryl oxidase is co-expressed in the *E. coli* cytoplasm (Hatahet et al. 2010). Finally, the correct multiple disulfide formation in the eukaryotic proteins in *E. coli* was improved by the co/pre-expression of the sulfhydryl oxidase

Erv1p with the combination of DsbC or PDI isomerase (Nguyen et al. 2011).

The N- and/ or O- linked glycosylation naturally cannot be performed in the *E. coli* cells. However, the utilization of heterologous glycosylation pathways allowed to obtain the glycosylation of several heterologous peptides in *E. coli* (Pandhal et al. 2010). For example, the introduction of the N-glycosylation pathway (encoded by *pgl*) from *Campylobacter jejuni* allowed to synthesize the glycolyzed *C. jejuni* proteins AcrA and PEB3 in *E .coli* host (Pandhal et al. 2010).

Recently, the same approach was employed for *in vivo* production of the ArcA glycoconjungates with the *Shigella dysenteriae* serotype-1-O antigen and exotoxin-A from *P. aeruginosa* (EPA). In addition, the reproducible *E. coli* fed-batch production process in the stirred-tank bioreactor for high level synthesis of the mentioned glycoconjugates was successfully developed (Ihssen et al. 2010).

Finally, the co-expression of the prokaryotic N (alpha)-acetyltransferase RimJ resulted in complete acetylation of the recombinant thymosin-α1 (Fang et al. 2009). In addition, the co-expression of the N-acetylation complex NatB from the fission yeast in *E. coli* cells resulted in the acetylation of the human Tropomyosin, Spartin, and yeast proteins Cdc8 (*S. pombe*) and Tfs1 (*S. cerevisiae*) (Johnson et al. 2010). Worth to mention, that protein acetylation is a very common modification in the eukaryotic cells (Arnesen et al. 2005).

2.2.7 Protein translocation and folding in *E. coli* periplasmic space

Naturally, protein translocation to the periplasmic space in *E. coli* cells could be driven via three secretion pathways/systems: Sec-dependent, Twin arginine (Tat) and signal recognition particle (SRP) (Mergulhão et al. 2008).

The Sec secretion pathway consists of the homotetrameric secretory cytoplasmic chaperon SecB, the inner membrane protein SecA and the membrane proteins SecYEG (du Plessis et al. 2011). The SecA

could be found soluble in the cytoplasmic space or peripherally associated with the integral proteins SecYEG (Collinson et al. 2005). The SecA directs pre-proteins to via the integral membrane pore SecYEG the protein for translocation across the membrane in an ATP hydrolysis-depended manner (Jilaveanu et al. 2005 and 2006, Tomkiewicz et al. 2006). The SecB chaperon maintains the secretion-prone proteins (pre-proteins) “folded” into so called translocation-competent state and delivers them to the SecA (Zhou et al. 2005). Interestingly, the cytoplasmic heat-shock proteins DnaK or GroEL/ES can substitute for the SecB during protein export (Wild et al. 1992 and 1996).

The pre-proteins possessing 20-30 partially hydrophobic amino acids and a downstream located cleavage site are recognized by the Sec secretory system (Marrichi et al. 2008). The cleavage of the target protein leader sequence occurs during the translocation by the inner membrane-associated signal peptidases Lep and LspA (Dalbey et al. 1991).

The signal recognition particle system (SRP) is composed of a GTPase (Ffh), 4.5 sRNA, and a membrane associated receptor Fsty (Bibi et al. 2011). The hydrophobic secretion leader peptide of the nascent protein is firstly recognized and bounded by the SRP. The formed complex is then binding the GTPase - Ffh. The released nascent peptide from the SRP and FstY (in GTP hydrolysis-depended manner) is translocated to the periplasmic space by the SecA and SecYEG complex (Wild et al. 2004, Schaffitzel et al. 2006).

The partially or completely folded proteins possessing the secretion tags with the conserved twin arginine motive are translocated via the “Twin arginine” depended (Tat) pathway. The Tat secretion pathway machinery is composed of four integral membrane proteins: TatA, TatB, TatC and TatE (Weiner et al. 1998, Müller et al. 2005).

The protein folding in the periplasmic space of *E. coli* is facilitated by a broad-substrate range generic chaperon Skp and the PPIase/chaperon activities possessing FkpA and SurA (Arié JP et al. 2001 and 2006, Sklar et al. 2007). Recently, the novel ATP-depended

periplasmic chaperon Spy was found which is also involved in the client protein folding and disaggregation (Quan et al. 2011).

The favourable for disulfide bond formation environment in the periplasmic space is created by the thiol-disulfide oxireductase system DsbA-G, members of which belong to the thioredoxin superfamily and possess Cys-X-X-Cys motif in the active site (Inaba et al. 2009). In the DsbA-G system, the periplasmic soluble oxidase DsbA (in the oxidized state) is responsible for the oxidation of the client protein cysteins (Akiyama et al. 1992). After the client protein oxidation, the reactive cysteins of DsbA turn into the reduced state. The reduced DsbA is re-oxidized by another inner membrane protein, DsbB (oxidase), which directly transfers the disulfide bonds to the reduced DsbA (Kishigami et al. 1995). After the disulfide bond donation the DsbB is also re-oxidized depending on the aerobic or anaerobic growth conditions by the ubiquinone or menaquinone, respectively (Inaba et al. 2008).

The reductase activity possessing protein DsbC is another DsbA-G system component, which catalyzes the reformation of “incorrectly” formed multiple disulfide bonds (Arredondo et. al 2009). The DsbC is maintained in the reduced state by the reductase DsbD (Bessette et al 1999). Besides the oxireductase activities, the DsbC and DsbG are acting as chaperons (Chen et al. 1999, Shao et al. 2000). Therefore, the co-expression of DsbC and DsbG facilitates the accumulation of heterologous proteins in soluble state (Zhang et al. 2002).

2.2.7.1 Strategies for periplasmic recombinant protein propagation in *E. coli* cells

Compared to the production in the cytoplasm approach, the heterologous protein propagation in the periplasm beneficial due to the presence of fewer proteases and “surrounding” proteins, easier release of the periplasmic protein (by an osmotic shock based cellular disruption), and the ability to generate disulfide bonds by the previously discussed Dsb machinery (de Marco A et al. 2009). In *E. coli* production platforms the heterologous protein translocation to the periplasmic compartment is mostly achieved by using the Sec-

depended N-terminal signal peptides, for example, MalE, OmpA, PhoA, PelB (Choi et al. 2004). The periplasmic translocation of heterologous proteins in *E. coli* could be also achieved by employing the leader sequences, which are recognized by the machinery of Tat secretion pathway. For example, twin-arginine signal peptide of TMAO reductase (TorA) served for the periplasmic translocation of the Green Fluorescence Protein (GFP) (Thomas et al. 2001, Santini et al. 2001 and Barrett et al. 2003), alkaline phosphatase and even a single-chain Fv and heterodimeric FAB antibody fragments (DeLisa et al. 2003). In addition, the efficiency of Tat-mediated translocation could be improved by modifying the secretion leader peptides (Medina-Rivero et al. 2007).

The common problems accompanying the periplasmic production are related with the cytoplasmic/periplasmic degradation, aggregation and inefficient translocation due to retainment in the membranes (Georgiou et al. 2005). The issues limiting periplasmic target protein propagation could be solved by applying various strategies: simply carrying the synthesis at lowered temperature (Balderas Hernández et al. 2008), and/or the co-expression and/or co-secretion of the cytoplasmic and periplasmic chaperons (Sonoda et al. 2011, Schlapschy et al. 2011).

The DsbA-G machinery often fails to facilitate the proper formation of the multiple disulfide bonds in *E. coli*. However, in some cases the disulfide bond isomeration in *E. coli* periplasm could be facilitated by using the SH groups-modifying low-molecular-weight folding-aiding cultivation medium additives –the combinations of the reduced/oxidized glutathione (GSH/GSSG) and/or arginine (Wunderlich et al. 1993 and Walker et al. 1994, Schäffner et al. 2001).

2.2.8 Proteolytic protein degradation in *E. coli* cells

2.2.8.1 Cytoplasmic proteases

The physiological role of proteolysis in the cell is to eliminate the incorrectly synthesized, misfolded, toxic proteins and short-lived

proteins involved in the regulation of metabolic events (Meyer et al. 2011). The enzymes possessing proteolytic activity are also attacking the heterologous proteins during the recombinant production and, thus, are causing a significant reduction in the recombinant product yield (Rozkov et al. 2004). Mainly, there are 5 cytoplasmic proteases, which could be involved in the recombinant protein degradation: Lon, ClpAP, ClpXP, ClpYQ, ClpB and the inner membrane- associated Fsth. All above mentioned proteases belong to the heat-shock response regulon and are depended on the ATP (Gottesman et al. 1996, Baneyx et al. 2004).

The Lon protease exists as a homo-hexameric enzyme (monomer of 87 kDa) (Botos et al. 2004, Rotanova et al. 2006). This multimeric protease is responsible for the proteolysis of abnormal, misfolded denatured proteins (Gur et al. 2008) and regulatory proteins, i.e. the division regulator (SulA) (Higashitani et al. 1997) and capsule synthesis regulator (RcsA) (Torres-Cabassa et al. 1987). The Lon protease is a stress-responsive enzyme, synthesis of which is stipulated by the heat shock and/or the recombinant protein production (Hoffmann et al. 2004). The cellular chaperons DnaK, GroEL and ClpB are unfolding/accommodating the client proteins for the degradation by Lon (Straus et al. 1988, Gottesman et al. 1997).

The protease complexes ClpAP and ClpXP are degrading the damaged unfolded/abnormal proteins (Singh et al. 2000, Maillard et al. 2011) and the client proteins tagged with an SsrA tag. In addition, the SsrA-tagged proteins are also attacked by Lon (Gottesman et al. 1998, Lies et al. 2008). The proteolysis of the SsrA tag-possessing proteins is enhanced by the modulator protein SspB (Levchenko et al. 2000, Wah DA et al. 2003, Lies et al. 2008).

The ClpAP and ClpXP comprise a serine protease domain ClpP and the chaperon/ATPase activities possessing ClpA and ClpX domains (Sharma et. al. 2005). The ClpA and ClpX serve for the substrate unfolding (Weber-Ban et al. 1999), recognition and accommodation for the cleavage by the ClpP protease in an ATP-depended manner (Singh et al. 2000, Hoskins et al. 2006, Lee et al. 2010, Maillard et al. 2011).

The HslVU (ClpYQ) is a complex ATP-depended protease comprising a protease domain HslV and an ATPase domain HslU. The multimeric protease domain HslV consists of 12-14 subunits (250 kDa) and exhibits a chymotrypsin-like activity stipulated by the ATP hydrolysis. The HslV exhibits weak peptidase activity when the ATPase domain is absent (Rohrwild et al. 1996, Azim et al. 2005). Multimeric HslU ATPase domain comprises 8-10 subunits (450 kDa) and also exhibits a chaperon activity, required for the substrate unfolding and translocation to the HslV (Park et al. 2005, Sundar et al. 2010, Baker et al. 2012). The HslVU mainly degrades SulA, RcsA (Kuo et al. 2004), δ^{32} and large abnormal proteins (Kanemori et al. 1997).

2.2.8.2 Periplasmic and membrane associated proteases

There are no ATP-depended proteases in *E. coli* periplasmic space, except the inner membrane-associated, ATP depended Zinc protease FstH (HflB). The ATPase and metalloprotease domains of FstH protease are associated to the cytoplasmic compartment (Akiyama et al. 2000). The FstH is involved in the cytoplasmic degradation of δ^{32} (Obrist et al. 2007, Meyer et al. 2011), some ssrA-tagged proteins (Herman et al. 1998) and membrane proteins (Akiyama et al. 1996).

The outer-membrane serine protease OmpT is responsible for the *in vivo* degradation of secreted recombinant proteins (Baneyx et al. 1990, Kandilogiannaki et al. 2001), soluble proteins after cell disruption in the crude extracts (Sedgwick et al. 1989), proteins during the protein purification (Grodberg et al. 1988) and even at the extreme denaturing conditions during the renaturation from inclusion bodies (White et al. 1995). The expression levels of OmpT protease increases in relation to the recombinant production, and in response to the heat shock (Gill et al. 2000). The OmpT protease is highly homologous to OmpP, which could be also involved in the recombinant protein degradation (Matsuo et al. 1999, Hwang et al. 2007).

The periplasmic protein DegP is inducible by the alternative heat-shock sigma δ^E protein. The DegP exhibits both –the proteolytic and the chaperon activities. At the low temperature DegP acts as a chaperon and at the higher performs as a protease (Kim et al. 2005, Meltzer et al. 2009). The DegP proteolytically degrades misfolded, abnormal periplasmic proteins (Krojer et al. 2008), including secreted recombinant proteins (Chen et al. 2004). The co-expression of DegP facilitates the recombinant protein folding (Pan et al. 2003) and reduces the periplasmic aggregation (Lin et al. 2001). However, the co-expressed DegQ and DegS, homologous to DegP, are incapable to facilitate the recombinant protein folding in *E. coli* periplasm (Pan et al. 2003).

2.3 Fed-batch production strategies

The fed-batch is the most commonly used cultivation methodology for obtaining the high cell densities This technique is based on the supply of the major nutrient source, mostly glucose or glycerol, in a controlled/limited manner, enabling to control the cell growth rate (Lee et al. 1996, Shiloach et al. 2005).

The feeding could be supplied in the constant mode or by using exponential feeding profiles. The closed loop feeding approaches are based on the substrate delivery control in relation to the fermentation parameters: dissolved oxygen level [DO-stat] and media pH [pH-stat] (Chen et al. 1997, Kim et al. 2004), concentration of the residual carbon source (glucose) and/or accumulated by- product (acetate) (Turner et al. 1994).

The specific growth rate is a highly important parameter for the recombinant production. The ideal specific growth rate μ in the pre-induction growth phase is considered to be from 0.1 to 0.25 h^{-1} (Curless et al. 1990 and 1994, Fan et al. 2005). The excessive substrate supply, stipulating the growth rate of μ~ 0.5 h^{-1}, is leading to the increased acetate formation and even ribosome destruction after the recombinant synthesis induction (Sandén et al. 2003). The uncontrolled nutrient inflow, exceeding the respiration capacity, is

inducing the overflow metabolism, thus, results in cell growth inhibition (Xu et al, 1999) and/or reduces the product formation level (Jensen et al. 1990, Xue et al. 2010), and/or enhances the target protein aggregation (Boström et al. 2005).

Cell starvation results in the inhibition of the product accumulation (Bentley et al. 1990, Flickinger et al. 1993), and the induction of the stringent and general stress responses (Teich et al. 1999)

The novel "mechanical pump-independent" substrate supply approach, where the feed rate is facilitated by the diffusion and/or biocatalysis, enables to create a stirred bioreactor-like fed-batch conditions in the microwell plates and/or shaken flasks (Jeude et al. 2006, Panula-Perälä et al. 2008). These novel techniques already proved to be a very useful tool for a high scale fed-batch process development, as they provide a possibility to evaluate the product expression system at the fed-batch mode in submillilitre scale and to find the optimal conditions for the production already at the very early developmental stages (Siurkus et al. 2010, Hortsch et al. 2010, Grimm et al. 2012). In addition, the enzyme-based substrate auto-delivery system EnBase® (Panula-Perälä et al. 2008) could be also utilized in a large scale rocking motion bioreactor for the high level fed-batch production of a heterologous protein (ADH) (Glazyrina et al. 2010). Besides auto-delivery of the substrate, the analogical systems could be utilized for the supply of other medium components, for example, pH regulating agents, like disodium carbonate (Scheidle et al. 2011).

No doubt, the tightly controlled substrate inflow, created by the auto-delivery techniques, are stipulating the efficient respiration and, thus, results in the favourable conditions for the recombinant protein accumulation in the soluble state and folding without the mechanical oxygen supply (Krause et al. 2010; Siurkus et al. 2010, Hortsch et al. 2011, Nguyen et al. 2011). In addition, the cell growth and specific productivity in the "mechanical pump-independent" fed-batch systems could be enhanced even more by increasing the dissolved oxygen levels in the cultivation medium, by supplying the oxygen from the oxygen-enriched agents, for example, liquid perfluorochemicals (PFCs) (Pilarek et al. 2011).

Alternatively, the substrate inflow could be controlled on the cellular level by utilizing the metabolic engineering approaches (Gosset et al. 2005 and Chou et al. 2007). For example, the inactivation of phosphoenolpyruvate: sugar phosphotransferase system (PEP-PTS) in the *E. coli* resulted in significantly increased GFP, recombinant TrpLE-proinsulin and β-galactosidase accumulation levels. In addition, the cell modifications resulted in lower acetate accumulation levels and even enhanced cellular growth (De Anda et al. 2006, Bäcklund et al. 2008, Wong et al. 2008). Interestingly, the reduction of substrate inflow by the inactivation of PTS also resulted in the accumulation increase of several His_6-tagged integral membrane proteins in *E. coli* B strain BL21 (DE3) (Bäcklund et al. 2011).

2.4 Ribonuclease inhibitor – function, features and heterologous production

The angiogenin ribonuclase inhibitors (RI or RNH1) are a unique family of the eukaryotic cytoplasmic proteins, possessing conserved/highly similar structural features, independent from the species of the protein host (Dickson et al. 2005). The RI has been isolated from the cells of various tissues from different hosts: liver of rat, bovine, pig, sheep and mouse (Burton et al. 1982), human placenta, erythrocytes (Blackburn et al. 1977, Nadano et al. 1994), testis (Ferreras et al. 1995), human, bovine and pig brain (Burton et al. 1980, Cho et. al 1989 and Moenner et al. 1998). Recently, RI was found in the mitochondria (Furia et al. 2011). The RIs form complexes, tightest among the known biomolecules, with the pancreatic type ribonucleases and angiogenin (Lee et al 1989, Kobe et al.1995). In the cell, the RIs are regulating the reactivity of the cellular ribonucleases, and protecting the cells against the invasive ribonuclease activity possessing enzymes (Shapiro et al. 2001). In addition, the RIs are acting as the redox state indicators and are involved in cell protection against the oxidative stress (Moenner et al. 1989, Furia et al. 2011).

The RIs are leucine-rich proteins, molecules of which comprise approximately 18 % of leucine residues from a total amino acid pool (Blackburn et al. 1977, Kobe et al. 1995). The horseshoe-like hydrophobic core of RI molecule is arranged from the β-strand and α-helix possessing structural units, known as leucine-rich repeats (LRR). There are two types of LRR's recognized in RI molecule: the typeA, containing 28 amino acid residues, and typeB, containing 29 residues. In each LRR subunit the β-strand and α-helix are connected by a loop and positioned in parallel to each other in the core of the molecule (Kobe et al. 1993). Depending on the origin, the RI molecule contains 30-32 cystein residues (approx. 7% from the total amino acid pool), which have to be in the reduced state in order to maintain the RI ability to inhibit RNases (Fominaya et al. 1992). Interestingly, the sequence analysis of several RI isolates from different mammalian hosts revealed 27 conserved cystein residues in all RI molecules (Dickson et al. 2005). Due to the RI exposure to the oxidizing factors, for example, 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB), the reduced cysteins undergo the sequential conformational changes. Firstly, the oxidation of one or more cysteins occurs, followed by the formation of mixed disulfide bonds. Finally, the oxidation of all cysteins yields a fully cross-linked, inactive form of RI (Fominaya et al. 1992). Approximately 50 % of the RI cysteins remain in the reduced state after the analogical treatment with DNTB when the RI is in the complex with the RNaseA. Partially oxidized RI is still capable to inhibit 75 % of the RNaseA activity. Worth to mention that *in vivo* the oxidative damage makes RI prone to proteolytic degradation (Ferreras et al. 1995). The resistance against the oxidation could be enhanced artificially by replacing the adjacent cysteins (Cys95A/Cys96A and Cys329/Cys330) with the alanine residues. After the sequence alteration RI maintains its ability to interact and inhibit the activity of RNaseA and ANG (see patent US5552302 and Kim et al. 1999).

There are several documented attempts to produce RI in heterologous host as a recombinant protein. The first RI production trails in *S. cerevisiae* and *E. coli* production platforms were not very successful due to an inefficient RI accumulation and/or a tendency to

aggregate into insoluble fraction (Lee et al 1989, Vicentini et al. 1990). However, the best yield of functional recombinant RI was achieved (approximately 15 mg L^{-1}) by using the *E. coli trp* promoter system (Klink et al. 2001). There is also a documented technique for the RI purification from the inclusion bodies, where the active RI was recovered from the solubilized IBs in the buffer contacting 4 M Urea and 5 mM of DTT. The RI refolding was accomplished by a rapid protein dilution with the DTT containing refolding buffer (see Promega patent US005552302A). In very recent reports RI was successfully accumulated in *E. coli* after the N-terminal fusing to the MBP tag (Siurkus, et al. 2010, Guo et al. 2011). The fed-batch production approaches based on the cytoplasmic redox control and the co-expression of the folding-facilitating chaperons allowed to achieve 320 mg L^{-1} of His_6-RI in *E. coli* K12 cells, and even 625 mg L of untagged RI in the T7 RNA polymerase construct (Siurkus et. al 2011).

2.5 Aim of research

The general aim of this study was to improve the accumulation of a highly aggregating, sensitive-to-oxidation model protein– the eukaryotic angiogenin ribonuclease inhibitor (RI), in *E. coli* product expression systems, and to develop the process for the propagation of sensitive-to-oxidation, aggregation-prone proteins. The general goal was subdivided into several sub-goals, in regards to the investigation phases:

- Evaluation of the influence of genetic factors (promoters, ribosome-binding sites and N-terminal fusion tags), growth mode and medium composition on the target protein cytoplasmic accumulation/folding. The process development and the scale-up of RI production as a fusion in the optimal expression system under the substrate-limited fed-batch cultivation mode;
- Evaluation of the impact of genetic factors (promoters, ribosome-binding sites and N-terminal periplasmic signal peptides) and low molecular weight medium additives on the RI periplasmic and cytoplasmic folding. The RI production at the substrate-limited growth conditions in the stirred-tank bioreactor by applying the developed folding approach.
- Evaluation of the cellular folding machinery bottlenecks, which are limiting the RI folding in *E. coli* cells.

3 Results

3.1 *E. coli* constructs utilized for RI propagation (papers I-III)

Initially, the RI was produced as a cytoplasmic protein by using the library of 45 expression vectors comprising systematic variation of promoters, ribosome-binding sites and following N-terminal fusion tags: His_6 (hexaxistidine), His_6SUMO (small ubiquitin-related modifier) His_6Trx (thioredoxin), His_6MBP (maltose binding protein MalE) and His_6GST (glutathion-S-transferase) (paper I, figure 1).

The RI propagation in periplasmic space of the *E. coli* was performed by using the parallel set of expression vectors harbouring the combination of analogical expression elements and combination of sequences for N-terminal periplasmic secretion leader peptides - *MalE*, *OmpA*, *PhoA* and *PelB* (for details see paper II and Kraft et al. 2007).

The vector libraries for the cytoplasmic and periplasmic RI expression were derived by utilizing modified gene expression regulatory elements from the vector pK100.

The utilized *lac* promoter derivatives - *lac_Cp*, *lac_CUp* and *lac_CTUp* and three ribosome binding sites of different strength – RBS (*T7, lac* and *var*) (paper I, figure 1) allowed to systematically vary target gene transcription and translation levels. A mutation in upstream element - CAP site and efficient transcription terminator – Hpt, allowed to reduce the target gene background and to ensure an efficient transcription control. Finally, both expression vector libraries were combined with the ColE1 pDest15 vector (from Invitrogen), enabling to insert the target genes by using Gateway® cloning technique via site-specific recombination into *λ* phage *attRI* and *attRII* sites (Kraft et al. 2006).

Prior to the co-transformation of the K12 RV308 strain with the expression vectors the cells were transformed either with the

pibpfxsT7lucA or the *plt* vectors to evaluate the cytoplasmic or periplasmic misfolding stress, respectively (paper I and paper II). In cytoplasmic misfolding stress reporter system the luciferase gene is controlled by the σ^{32}-dependent tandem promoters - *ibpAB* and *fxsA*, which are activated in reponse to cytoplasmic aggregation (see paper I). Analogically, in periplasmic stress reporter the luciferase gene expression is driven by the σ^{24}-dependent *degP* promoter, which is induced in response to elucidated by the periplasmic aggregation stress (for details see paper II and Kraft et al. 2007).

Finally, for the cytoplasmic RI propagation in T7 RNA promoter constructs the native RI gene was cloned into the pET21b vector ((see paper III). For the chaperon co-expression in *E. coli* ER2566 pET21b-RI construct the cells were co-transformed with the GroEL-GroES and DnaK-DnaJ-GrpE chaperon genes carrying vectors - *pGro7* and *pKJE7* (Takara Bio Inc), respectively (paper III, “Materials and Methods” section).

3.2 High throughput screening for optimal RI cytoplasmic production construct (paper I)

The screening for optimal cytoplasmic RI production was performed during the RI propagation under the fed-batch mode in 45 cytoplasmic expression constructs, possessing cytoplasmic folding stress reporter *pibpfxsT7lucA.* Bacteria cultivation/protein expression was carried in 150 µL of mineral salt medium (MSM) in a 96 well format microwell plate. The fed-batch mode in microscale was generated by using a substrate autorelease technology Enbase® (Peralla et al. 2008). In order to avoid a very high variation in cell densities resulting after the plate inoculation directly from the glycerol stocks, an additional 12 hour microwell-batch cultivation stage was included. After the synchronization step, the fed-batch cultivation was initiated by supplementing the medium with required amount of glucose auto-release stipulating enzyme and carried for another 12 hours at 37 ℃ (for technical details see paper I “Materials and Methods” section). After the preparation of inoculum the synchronized constructs were

transferred to a new cultivation plate and further cultivated under the fed-batch mode until RI synthesis induction point corresponding to OD_{600} of 12 ± 1.0 OD_{600} was reached. The RI synthesis was carried for 7 h at 37, 30 or 22℃ (see paper I, figure 2). It was considered, that RI production constructs had highly similar physiological state due to similar growth rate at the induction point, thus the evaluation of genetic factors for RI folding and accumulation was objective. After the RI fed-batch synthesis course at the above listed temperatures the intensities of luminescence signals were measured in order evaluate the constructs/synthesis conditions which served the best for tagged RI accumulation in the soluble protein fraction. In addition, at the same time the cells were harvested for the analysis of total soluble and insoluble protein fractions by using 10 % SDS-PAGE and Agilent 2100 bioanalyzer LabChip techniques (paper I, “Materials and Methods” section).

The obtained luciferase expression levels, which are tightly related with the target protein cytoplasmic aggregation, were highly similar after the tagged RI production at all temperatures tested. The constructs with the expression vectors, in which RI was fused with His_6, His_6-GST, His_6-Sumo, or His_6-Trx, produced the highest luminescence signals. Only the constructs, in which RI was propagated in the fusion with His_6-MBP, produced 2-3 fold lower luminescence levels, independently from the propagation temperature and utilized genetic elements (paper I, figure 3 and supplementary figure 1). In addition, all constructs produced insignificantly lower luminescence signals after the tagged protein production at 30 and 22 ℃ (paper I, figure 3 A and supplementary figure 1) .

The analysis of soluble protein fraction showed that only His_6-MBP-RI fusion protein was efficiently accumulated in the soluble protein fraction regardless of the gene expression regulating elements and synthesis temperature (paper I, figure 3). Contrary, in all expression constructs, producing high level luminescence signals, the His_6-RI, His_6-GST-RI, His_6-Sumo-RI and His_6-Trx-RI were highly aggregating (paper I, figure 4). Finally, protein analysis revealed, that the lower production temperature reduced tagged RI and/or luciferase

expression levels and, thus, the luminescence levels were observed lower compared to the signals obtained after the synthesis at 37 ℃ (paper I, figure 3).

In summary, at the screening stage it was found that (i) the promoters and/or ribosome-binding-sites are not significant factors for tagged RI soluble accumulation, and that (ii) soluble RI accumulation is very depended on the His_6-MBP N terminus tag. Therefore, in the second work phase the evaluation of the cultivation/induction conditions for the RI accumulation in the larger scale was performed by using the construct harbouring strong expression elements: *pCTU* promoter, *T7*-ribosomal-binding site and N-terminal His_6-MBP tag (paper I, “Results” section).

3.3 Evaluation of cultivation factors influence on His_6-MBP-RI accumulation (paper I)

In this part the impact of cultivation mode, specific growth rate and composition of cultivation medium on the His_6-MBP-RI accumulation in *E. coli* RV308/*pibpfxsT7lucA*/pCTUT7MBP-RI construct was evaluated.

The substrate auto-delivery system Enbase® allowed to perform His_6-MBP-RI synthesis induction at different specific growth rates. Therefore, the most suitable fed-batch growth mode for target protein synthesis induction could be revealed. The fed-batch cultivation with recombinant synthesis was performed in 200 mL of MSM medium in 1 L cultivation flasks (paper I, “Materials and Methods” section). The His_6-MBP-RI synthesis induction was performed at the following specific growth rates and cell densities: t_1 = 4 h, $OD_{600\ (t1)}$= 2.0 ± 0.2, $\mu_1 \approx 0.33\ h^{-1}$; t_2 = 8 h, OD_{600} = 5.5 ± 0.2, $\mu_2 \approx 0.22\ h^{-1}$; and t_3 = 13 h, OD_{600} = 11 ± 0.2, $\mu_3 \approx 0.1\ h^{-1}$. In all fed-batch trials the His_6-MBP-RI production was performed for 4 hours at 37℃ (paper I, figure 5).

The batch His_6-MBP-RI synthesis in the same construct was initiated at OD_{600} of 1 in glucose-MSM medium ($\mu \approx 0.38\ h^{-1}$), LB, and semi synthetic medium ($\mu \approx 0.7\ h^{-1}$). Analogically to fed-batch experiments,

the target protein synthesis was continued for 4 h at 37 ℃ (paper I, “Materials and Methods” section).

The protein analysis revealed that the specific growth rate of pre-induction phase and medium composition has a huge impact on soluble His_6-MBP-RI accumulation. The most favourable induction conditions, yielding approximately 38 mg per gram of cell dry weight of His_6-MBP-RI (corresponding to approx. 130 kU per gram of wet cell weight),were found when the induction was carried out under the fed-batch growth mode in MSM medium at the specific growth rate of $\mu \approx 0.22\ h^{-1}$. The fed-batch synthesis after induction at $\mu \approx 0.33\ h^{-1}$ and $\mu \approx 0.1\ h^{-1}$ gave by 15 % and 55 % lower yield of the total His_6-MBP-RI , respectively, compared, to the best fed-batch synthesis result. Interestingly, the His_6-MBP-RI accumulation level after the induction at batch growth ($\mu \approx 0.38\ h^{-1}$) in the same glucose MSM medium was obtained by 12 and 32% lower, compared to the fed-batch synthesis trails with induction at $\mu \approx 0.33\ h^{-1}$ and $\mu \approx 0.22\ h^{-1}$, respectively (paper I, figure 6). The highest aggregation levels of His_6-MBP-RI and, thus, the lowest amount in the soluble protein fraction were detected after the recombinant synthesis in MSM medium, supplemented with the complex additives and “classic” LB medium (paper I, figures 6 and 7).

3.4 His_6-MBP-RI production in batch and fed-batch bioreactor (paper I)

The His_6-MBP-RI production experiments in stirred-tank bioreactor were designed by taking into account the results derived from the shake flasks production trails. The feeding profile in fed-batch substrate limited processes were designed to maintain the specific growth rate of $\mu \approx 0.22\ h^{-1}$, which was considered as an optimal for the target protein synthesis induction (see paper I, “Materials and Methods” section). In addition, in order to evaluate the effect of cell densities on the His_6-MBP-RI accumulation, the target protein synthesis induction was performed at OD_{600} of 9 and 31 at the same specific growth rate (paper I, figure 8). The His_6-MBP-RI synthesis induction under the batch growth in stirred-tank bioreactor was

performed at the logarithmic growth phase corresponding to $\mu_{max}$of 0.45 h^{-1} (paper I, figure 8). Worth to mention that synthesis induction of the target protein in all bioreactor processes was initiated by using the same inducer concentration, and synthesis was continued at 37 ℃ for 4 hours in glucose-MSM medium (paper I, “Materials and Methods” section).

The His_6-MBP-RI production under the fed-batch in stirred-tank bioreactor gave highly similar patterns of the total and soluble RI accumulation, independently from the cell densities. In addition, the RI accumulation patterns after the production in the Enbase-shaking-flasks were obtained highly similar to the results from stirred-tank bioreactor production, when the induction was carried out at the specific growth rate μ of 0.22 h^{-1} (paper I, figure 9). Interestingly, the batch production process in stirred-tank bioreactor gave unexpected soluble amount of His_6-MBP-RI by 40% lower, compared to the best fed-batch production process and lower by 5 % compared to the analogical process in batch shaking flasks (paper I, figure 9).

3.5 High throughput screening for optimal RI periplasmic production construct (paper II)

The screening for the efficient RI periplasmic production constructs was performed by using the above described set of 36 periplasmic expression vectors. The *plt1* plasmid was used to evaluate the periplasmic misfolding stress (paper II, “Material and methods” section).

The evaluation of the RI periplasmic accumulation was performed after 5 h of the recombinant expression under fed-batch cultivation mode in Enbase® 96 microwell plates at 22 ℃ in MSM cultivation medium. Prior to induction the constructs were cultivated at 30 ℃ for 12 hours until OD_{600} of 12 ± 2 (μ ≈ 0.16 h^{-1}). Worth mentioning, that in this screening experiment the periplasmic RI production was carried out after direct inoculation of microwell plate with the glycerol stocks containing highly similar cell amounts. The stocks were produced by

overnight cell cultivation in the shake flasks (paper II, “Materials and Methods” section).

Similar to previously described microscale cytoplasmic screening results, all the constructs resulted in high RI accumulation level. However, the RI accumulation in constructs harbouring strong expression elements was achieved by 20-30% higher, compared to the constructs possessing a combination of weak promoters (*pC, pCU*) and ribosome-binding-sites (*var*, and *lac*) (The SDS-PAGE images not showen). In addition, after the RI periplasmic production an interesting relation between the cell growth at the post induction phase and the utilized expression vector elements was revealed. The growth of constructs harbouring strong expression stipulating elements was completely inhibited after the synthesis induction, compared to the constructs possessing weaker expression elements (paper II, figure 1).

The produced luminescence signals were not related with the strength of the expression elements, RI periplasmic accumulation and aggregation levels. Interestingly, the group of constructs harbouring vectors with weaker expression elements produced by 2-3 fold higher luminescence signals, compared to the strong expression elements possessing constructs (see paper II, figure 1). Despite the low production temperature, RI was highly aggregating, thus, the accumulated levels of soluble RI were extremely low in cytoplasmic space and not detected in periplasmic compartments of all production constructs (SDS-PAGE images are not showed).

3.6 RI folding in *E. coli* periplasm (paper II)

In this part of the work the main focus was directed towards the significance of cysteins for RI folding in *E. coli* K12 periplasmic and cytoplasmic compartments. Therefore, the initial RI periplasmic production experiments were performed by using periplasmic oxireductase DsbA deficient strain RV308*dsbA*$^{-}$ and isogenic “original” RV308 strain (paper II, Materials and Methods section). The two sets of vectors harbouring weak (pCU lac and/or var, and leader peptides:

MalE, OmpA, PhoA and PelB) and strong (pCTUT7- MalE, OmpA, PhoA and PelB) expression stipulating elements were used for RI periplasmic propagation under reducing conditions.

The reducing conditions to facilitate the RI periplasmic folding were created by supplementing the medium with 20-50 mM of reduced glutathione or 2-18 mM of DTT. The periplasmic RI propagation was performed at 22 ℃ under the batch cultivation mode . The required amount of GSH or DTT was added at the same time point as IPTG, in tandem with the cultivation temperature downshift (paper II "Materials and Methods" section).

The accumulation of RI in total, soluble and insoluble fractions of the periplasmic constructs was analyzed by 10 % SDS-PAGE. The RI activities in total soluble and periplasmic protein fraction were determined by ribonuclase inhibitor activity assay (see paper II, "Materials and methods" section). The results showed that the reduced GSH did not have any obvious impact on the RI soluble accumulation in the constructs harbouring strong and weak expression elements in *dsbA* deficient and isogenic strains (the gel images and graphs not shown). In addition, the medium supplementation with DTT did not provide a desired RI soluble accumulation and/or activity improvement in strong expression elements: *pCUT7- malE, -ompA, -phoA,* and *–pelB* possessing constructs. However, the DTT highly improved the periplasmic RI accumulation in *dsbA* deficient and isogenic strain constructs harbouring weak expression elements (*pCUvar - malE, ompA, phoA* and *pCUlac -pelB*). The best production results of periplasmic RI were achieved after medium supplementation with 12-18 mM of DTT in *dsbA* positive constructs and 6-12 mM in *dsbA* deficient constructs (see paper II, figures 2, 3 and 4). Worth mentioning, that the *dsbA* gene deficient constructs exhibited a lower DTT tolerance, thus, the accumulation levels of RI in the total protein fraction was achieved lower too, compared to the isogenic constructs (see paper II, figures 2 and 3). However, after all production experiments with or without DTT in the medium, the RI activities in the periplasmic fraction of *dsbA* deficient cells were detected higher by 40-60 %. The RI total activities

were obtained higher by 20-40 % in the constructs possessing intact *dsbA* gene (see paper II, figure 4). Finally, the analysis of protein fractions revealed that DTT positively affected the accumulation of unprocessed RI in the soluble fraction of cytoplasmic space in all tested constructs (paper II, figure 3).

3.7 DTT mediated RI folding in *E. coli* cytoplasm (paper II)

In order to evaluate the DTT effect on RI folding in *E. coli* cytoplasm, the set of batch cytoplasmic production experiments were performed at 22 ℃, 30 ℃, 37 ℃ and medium supplementation w ith various amounts of DTT (2-18 mM) (paper II, “Materials and Methods” section). In all cytoplasmic production experiments the RV308 pCUlac His_6-RI construct was utilized (see paper I). Worth mentioning, that tested RI cytoplasmic propagation construct was harbouring the same weak expression elements (*pCU-, lac-*) as the best performing periplasmic construct (paper II, “Materials and Methods” section). The protein analysis revealed a huge impact of amount of added DTT and the production temperature on His_6-RI accumulation in the soluble protein fraction (paper II, figure 5). The data showed that, most favourable production conditions for His_6-RI cytoplasm accumulation and activity were obtained due to low production temperature and highly reducing conditions, which were created by 12-18 mM of DTT in the cultivation medium (see paper II, figure 5). Interestingly, the periplasmic production under the same conditions resulted 4-5 fold lower RI activities compared to the best result obtained after analogical cytoplasmic production (see paper II, figure 4 and figure 5).

3.8 Periplasmic and cytoplasmic RI folding under fed-batch production growth in the shake flasks (paper II)

Prior to the fed-batch trails in the stirred-tank bioreactor, the performance of dsbA$^{-/+}$ periplasmic (RV308 *dsbA*$^{+}$ pCUlac pelB-RI and RV308 *dsbA*$^{-}$ pCUvar malE-RI) and cytoplasmic (RV308 pCUlac-His$_6$-RI) production constructs were evaluated under substrate-limited growth mode. The substrate-limited mode in the shake flasks was generated by using the Enbase® cultivation technology (paper II, "Materials and Methods" section). Analogical to the batch trials, the required amount of DTT to the cultivation medium was added in tandem with IPTG and downshift of cultivation temperature. In all fed-batch production trails the RI synthesis was induced at the substrate-limited growth, corresponding to OD_{600} of 5 ± 0.5, $\mu \approx 0.22\ h^{-1}$. The expression of target protein was carried out for 4 hours at 22 ºC, without (as a control) and with 12 mM of DTT in the cultivation medium (paper II "Materials and Methods" section).

The analysis of results revealed, that DTT resulted 2.6 fold increase of RI activities compared to the analogical synthesis without DTT in non modified periplasmic RV308 *dsbA*$^{+}$ pCUlac pelB-RI and cytoplasmic RV308 pCUlac His$_6$-RI constructs (see paper II Figure 6). However, the *dsbA*$^{-}$ strain showed very poor growth at the fed-batch mode and exhibited very low expression of target protein level, especially after medium supplementation with DTT (results not showed). In addition, in response to DTT the RI expression levels were 15-20 % decreased even in *dsbA*$^{+}$ constructs, compared to the analogical synthesis under batch mode (paper II, figures 5 and 6).

3.9 Periplasmic and cytoplasmic RI folding in the stirred-tank bioreactor (paper II)

The fed-batch process for RI production in the stirred-tank bioreactor was designed by considering the RI production results under substrate

limited mode in the shaking flasks. Thus, the RI synthesis induction was performed at the substrate limited growth mode corresponding to μ of 0.22 h^{-1} at OD_{600} of 28 (see paper II, figure 8). In all fed-batch processes, the constant growth rate was maintained by exponential feeding (see paper II, “Materials and Methods” section). In addition, in all production trails the cultivation temperature was downshifted from 37 to 22 ℃ at target protein synthesis induction (IPTG addition) point and maintained during the whole recombinant production course.

The periplasmic and cytoplasmic RI production during the batch-type cultivation was induced at exponential growth phase corresponding to OD_{600} of 7 ($\mu \approx 0.45\ h^{-1}$) and carried at 22 ℃ for 4 hours (see paper II, “Materials and Methods” section).

In the first batch-type bioreactor production trails the 12 mM of DTT was added as single pulse in tandem with IPTG. In addition, in order to prevent rapid oxidation of DTT by dissolved oxygen (pO_2~30% during culture growth), prior to addition of DTT, the air flow was drastically reduced from 30 L min^{-1} to 2 to 3 L min^{-1} , thus, actual oxygen concentration in the cultivation medium was maintained close 0 % (see paper II “Materials and Methods” section). However, this strategy of induction resulted in loss of target protein accumulation (results not shown). Therefore, in the following trails the first pulse of DTT and downshift of aeration was performed separately from target protein induction and temperature downshift (see paper II, figure 8). However, despite the recovered RI accumulation, the improved RI production approach allowed to achieve only a minor 1.4 fold increase of cytoplasmic and periplasmic RI activities compared to control processes (paper II Figure 8).

Worthy of note is that even limited aeration generated more than 50 % of oxidized DTT during 3 hour synthesis course. The oxidation rate of DTT in the shake flasks was indicated significantly lower - 5-10 % after 4 hour production course at 22 ℃ (paper II F igure 9).

Therefore, in the third set of batch and fed-batch experiments in the stirred-tank bioreactor, the repeated DTT pulsing approach was tested (paper II, “Materials and Methods” section and figure 8). Contrary, to the results obtained after production of RI with a single DTT pulse, the

repeated addition of DTT at the oxygen limited conditions resulted in significant (2-3 fold) increase of RI periplasmic and cytoplasmic activities after batch and fed-batch synthesis (paper II, figure 8). Worth to mention, that batch production with repeated supplementation of DTT resulted in 10-15 % higher RI cytoplasmic and periplasmic activities per cell, compared to the analogical production experiment at the substrate-limited mode (paper II, figure 8).

3.10 The RI production in T7 RNA polymerase system (paper III)

The production of RI possessing native sequence in T7 RNA polymerase construct ER2566 pET21b was performed in order to (i) evaluate redox effects on RI folding in the strong elements possessing construct and to (ii) find bottlenecks in the cellular folding machinery, which are limiting soluble accumulation of RI. Initially, for RI production in T7 RNA constructs the batch production protocol described in paper II was applied. The RI synthesis was carried at 22, 30 and 37 ℃ for 4 hours, after synthesis induction with 0.2 mM of IPTG. The medium supplementation with 12 mM of DTT was carried out at the synthesis induction point or 2 hours after synthesis induction. The aim of “delayed” DTT addition was to reduce possible physiological stress elucidated by combination of strong synthesis induction temperature, downshift and sudden redox alteration (paper III, “Materials and Methods” section).

The RI production in T7 RNA polymerase system results revealed, that medium supplementation with DTT did not positively affected accumulation of soluble RI nor improved activity per cells, independently from production temperature or DTT addition time (paper III, figure 1 and figure 2). Therefore, the analogical RI production trails were carried out with the co-expression of the main *E. coli* chaperons groups – DnaK/J/GrpE or GroEL/ES (paper III, “Materials and Methods” section). The co-expression of the DnaK/J/GrpE resulted in improvement of the RI accumulation in the soluble fraction, but not the activities per biomass independently from

synthesis temperature and/or DTT addition mode. Contrary, the co-expression of GroEL/ES chaperon group resulted in a significant improvement of RI solubility and activity, even without medium supplementation with DTT. In addition, the results showed, that GroEL/ES mediated RI folding is highly depended on the synthesis temperature (see paper III, figure 2 and figure 3). The DTT pulse in combination with the co-expression of GroEL/ES resulted in further 30-55% improvement of RI soluble accumulation and 1.5-2 fold increase of RI activity per cells compared to the analogical batch process without DTT (paper II, figure 1 and figure 2). In summary, the results revealed that the conditions favourable for propagation of active RI in T7 RNA polymerase expression systems were created due to the GroEL/Es co-production, production at 22 ℃ and medium supplementation with 12 mM of DTT (paper III, figure 1).

Worth mentioning, that DTT addition time had an insignificant impact on the volumetric activity and quantity of soluble RI in the GroEL/ES co-expressing T7 RNA polymerase construct. The highest amount of soluble RI was obtained after production process based on “delayed” medium supplementation with DTT. However, the DTT addition at the synthesis induction point resulted in higher RI activity per cells (see paper III, figure 1 and figure 2).

Finally, the analysis of results revealed an interesting relation between the total RI accumulation level, synthesis temperature and DTT addition time. In all production trails were DTT addition was performed at the RI synthesis induction point and synthesis was carried at 37 ℃, the total RI accumulation per cell was drastically decreased (paper III, figure 2). Interestingly, when the RI synthesis was carried out at the low production temperature - 22 ℃ and/or DTT was supplied 2 hours after synthesis induction the accumulation of total RI was not affected (paper III, Figure 2).

3.11 The GroEL/ES and redox mediated RI folding in T7 RNA polymerase system under fed-batch growth mode (paper III)

3.11.1 Fed-batch process in shake flasks

By taking into account the lessons learned during the development of RI fed-batch production processes described in the papers I and II, the process based on co-expression of chaperonins, and modulation of redox state was verified in the shaking flasks under substrate limited mode by the Enbase® cultivation technique (paper III, “Materials and Methods”).

In all fed-batch production trials the RI synthesis was carried out for 4 hours at 22℃. The RI accumulation with GroEL/ES chaperons was evaluated after synthesis induction at two fed-batch growth modes: $OD_{600} \approx 5$ ($\mu \approx 0.22\ h^{-1}$) or $OD_{600} \approx 11$ ($\mu \approx 0.10\ h^{-1}$). The DTT was added either at the synthesis induction point or 2 hours after synthesis induction (paper III, “Materials and Methods” section). The analogical fed-batch production trials without medium supplementation with DTT were included as the controls.

Differently from the analogical batch production trials, the DTT pulse in tandem with synthesis induction resulted in very low RI accumulation, independently from the growth rate at the induction time point (results not showed). Even the delayed DTT pulse resulted in significant - 50 to 70 % decrease of total RI accumulation when synthesis induction was carried out at $\mu \approx 0.22\ h^{-1}$ and $\mu \approx 0.10\ h^{-1}$, respectively (paper III, Figure 3). Despite the decrease of the total RI accumulation, the combination of delayed DTT addition with co-expression of the GroEL/ES resulted in 2 fold improvement of the RI activity per cells, especially when synthesis was induced at $\mu \approx 0.22\ h^{-1}$ (paper III, figure 3). Worth to mention that optimal RI production process under fed-batch growth mode yielded approximately five fold higher volumetric activity of RI, compared to the analogical batch production process.

3.11.2 The RI production with GroEL/ES chaperon co-expression and redox control in stirred-tank bioreactor (paper III)

The fed-batch RI production experiments in the shake flasks revealed the key induction and synthesis parameters. The RI production process with chaperon co-expression and redox state modification in the stirred-tank bioreactor was designed by considering those results. The previously described RI production in the fed-batch bioreactor setup (paper II), was also used for RI propagation in T7 RNA system. Prior to the RI production trails under substrate-limited mode, several experiments with co-expression of GroEL/ES and DTT pulsing (single and repeated) were conducted in the batch-type bioreactor. In all batch-type production trials the induction of RI synthesis was performed at the exponential growth phase at OD_{600} of 5 ($\mu \approx 0.5\ h^{-1}$) and continued for 5 hours at 22 ℃. The batch pro cess without medium supplementation with DTT was performed and considered as the control for evaluation of the redox state significance for RI activity and accumulation level in T7 RNA construct.

The RI production under substrate limited mode was induced at OD_{600} of 20 or 38, at the specific growth rate μ of $0.22 \pm 0.03\ h^{-1}$, which was maintained by exponential glucose feed supply (paper III, “Materials and methods” section). Analogical to the batch process, the RI fed-batch production was continued for 5 hours at 22 ℃ under continuation of exponential feeding. In all bioreactor processes the chaperon co-expression was induced approx. 1 hour before RI induction at 37 ℃. In addition the single or repea ted DTT pulses were started 2 hours after induction of RI, i.e. immediately after down-regulation of air flow from 30 to 3 - 5 L min^{-1} to maintain the dissolved oxygen concentration in the cultivation medium close to 0% (paper III, figure 4 and “Materials and Methods” section).

The results obtained after RI production in the stirred-tank bioreactor showed, that in all batch and fed-batch production processes the single and repeated DTT pulse reduced total RI accumulation by a level of 30 to 40%, compared to the analogical processes, conducted

without DTT supplementation (paper III, figure 5). However, depending on the single or repeated pulsing of DTT, the soluble RI accumulation during fed-batch production was improved by 30 to 35 % and activities per biomass by 3.2 - 4 folds, respectively (paper III, Figure 5). Interestingly, contrary to the previously described RI production processes in the stirred-tank bioreactor, where K12 strain RV308 constructs was used (paper II), in GroEL/ES T7 construct even a single DTT pulse resulted in significant RI activity improvement per cells. This was unexpected, because the DTT oxidation pattern after synthesis course under low aeration conditions was observed highly similar to *E. coli* K12 process, corresponding to 50-60% of oxidized DTT in the stirred-tank bioreactor and 10-15 % in the shake flasks. In addition, contrary to the K12 strain process, the ER2566 constructs was still growing at $\mu \approx 0.11$- $0.15\ h^{-1}$ even during DTT pulsing (paper III, figure 4).

In summary, the fed-batch production process based on the repeated DTT pulsing and the co-expression of GroEL/ES produced 625 mg of soluble RI per litter of culture medium. That corresponds to RI volumetric activity of approximate 80,000 kU L^{-1} (paper III, “Results” section).

3.12 Comparative proteome analysis of K12 RV308 strain construct after RI cytoplasmic production (paper IV)

The aim of the conducted comparative proteome analysis (CPA) was to elucidate protein groups in RV308 pCU lacHis_6RI construct, which are induced in response to multiple stresses caused by: (i) cytoplasmic over-expression of the heterologous protein (ii) temperature decrease (ii) cultivation under oxygen limitation conditions, and (iii) medium supplementation with 12 mM of DTT.

The samples for CPA were produced from two types of cultivation/RI production: (i) shake flasks and (ii) stirred–tank bioreactor.

The RI production in the shake flasks with redox modulation was performed as described in paper II, “Materials and methods” section. The RV308 pCU lac His_6RI construct was cultivated as a batch culture at 37℃ and 180 rpm in MSM medium containing 10 g L^{-1} of glucose until the induction point was reached, corresponding to cell density of OD_{600}= 1 ± 0.05 ($\mu \approx 0.35$ h^{-1}). The RI synthesis was induced by addition of 1 M IPTG to the final concentration of 0.2 mM. The reducing agent DTT was added to the cultivation medium at the time of RI induction as a dry powder to achieve final concentration of 12 mM. Cytoplasmic expression was carried out for 3 h at 22 ℃ at the shaking rate of 180 rpm. The cell samples for 2DE electrophoresis runs were taken 10 minutes before RI synthesis induction (“Master Sample – “[-10 min sample]”) and 3 hours after (“Stressed sample” – [+180 min]) (paper IV).

The RI production in stirred-tank bioreactor was performed as described in paper II. The RI synthesis induction was performed at the fed-batch growth mode OD_{600} of 7± 0.5 ($\mu \approx 0.22$ h^{-1}) which was generated by exponential feeding. The temperature down-shift from 37 ℃ to 22 ℃ was carried-out at the same time as RI synthesis induction. The RI production was continued for 5 hours at 22 ℃ under glucose limited growth mode. Prior to medium supplementation with DTT concentrate the air flow was down-regulated from 30 % pO2 for maintenance of pO_2 close to 0 % (see paper II). Immediately after establishment of low-aeration conditions, i.e. 2 hours after induction, the cultivation medium was supplemented with DTT concentrate to achieve final concentration of 12 mM of DTT in the cultivation medium. The synthesis was continued for another 3 hours under exponential feeding mode and low-aeration conditions (paper IV, “Materials and Methods” section).

The non-stressed sample for CPA was harvested 10 minutes before synthesis induction after cell cultivation under substrate-limited mode (pO2 30 %, 37 ℃). The sample representing the “str essed culture” [+300 min] was harvested 300 minutes after RI synthesis under fed-batch mode i.e., 180 minutes after initiation of low aeration conditions and addition of DTT (paper IV). The samples for CPA analysis were

derived from 3 independent cultivation experiments and 2 2DE gels from each experimental point.

The methodologies for sample, preparation, selection of protein spots and data analysis were performed as described in paper IV, “Materials and Methods” section.

The CPA revealed 57 up-regulated proteins after RI hour RI production in the batch-type shake flasks and 59 proteins were identified after 5 hours of RI synthesis in the glucose limited fed-batch cultivation (paper IV, “Result” section).

The identified proteins after RI production in the batch-shake flasks were classified into 8 categories according to their physiological roles in the *E. coli K12* cells. The proteins indicated after 5 hours of RI synthesis in the glucose- limited fed-batch bioreactor were classified into the 7 categories (Paper IV).

3.13 The utilization of DTT for folding of various prokaryotic and eukaryotic origin proteins (unpublished data)

In order verify the DTT as versatile recombinant protein folding facilitator in various *E. coli* expressions constructs the batch synthesis trials of RpoB-lysC (200 kDa fusion protein), DnaseI from Bovine pancreas, thioredoxin, light chain enterokinasefusion protein (TrxA-EK) and SssI methyltransferase from *Spiroplasma sp.* were carried with and without medium supplementation with 15 mM of DTT.

3.13.1 The DTT mediated folding improvement of of highly aggregating fusion protein RpoB-lysC

The RpoB-lysC is a 200 kDa fusion protein, derived by fusing of RpoB – *E. coli* RNA polymerase β subunit with lysC – *E.coli* aspartate kinase III. The RpoB – lysC fusion protein possessing 12 cysteins and is highly aggregating during recombinant synthesis in *E. coli* JM109 pASK-IBA construct at 30 ℃ (figure 1). In order to verify the DTT effect on RpoB-lysC accumulation two analogical batch synthesis

trials with and without DTT supplementation (as a control) were performed (described below).

The inoculum cultures for RpoB – lysC production in the shake flasks were prepared by overnight batch cultivation of the construct in 500 mL shake flaks with 50 ml of LB medium. For protein production 5 % of inoculum culture was transferred to fresh LB medium to the final volume of 500 mL in 2 L baffled Erlenmeyer shake flasks. Cultures were cultivated at 37 ℃ and 180 rpm until reached the induction point corresponding to a cell density of OD_{600}= 0.7 ± 0.05 ($\mu \approx 0.5\ h^{-1}$). Induction was performed by addition of 2 mg/mL anhidrotetracycline (aTc) to a final concentration of 0.2 mg/L. The DTT was added to the cultivation medium at the RpoB-lysC synthesis induction point as dry powder to achieve the needed concentration of 15 mM. Cytoplasmic expression was carried for 4 h at 30 ℃ at a shakin g rate of 180 rpm.

After synthesis, harvested cell samples were resuspended in lysis buffer with the following biomass to buffer ratio: 1 g of biomass with 10 mL of lysis buffer (50 mM Tris-H_3PO_4 pH 8.0, 0.1 % Triton X-100, 2 mM EDTA, 1 mM PMSF, 1 mM DTT and 0.1 mg mL^{-1} lysozyme). After 30 min of lysis at +4 ℃ the biomass was disrupted by sonication for 60 sec (Vibra cell™, Sonic and Materials Inc., 6 mm diameter probe tip) at +4 ℃. Soluble and insoluble protein fractions were se parated by centrifugation for 30 min, max g, at $+4^{0}$℃. After centrifugation the insoluble protein pellet was additionally washed and resuspended in the original volume of lysis buffer without lysozyme.

Samples for SDS-PAGE separation were prepared as follows: 20 µL of protein sample (total soluble, insoluble, protein suspensions), 25 µL of 4×SDS-PAGE loading buffer (Thermofisher scientific), 5 µL of 20×DTT (Thermofisher scientific) and 50 µL of deionised water to obtain a final sample volume of 100 µL. Samples were heated for 15 min at 95 ℃. 10 µL of sample was applied to each lane of 8 % SDS-PAGE gel. Differences in the amounts of RpoB-lysC in soluble fractions after productions with and without DTT were evaluated from scanned SDS-PAGE gel images by image treatment with TotalLab software.

The results analysis revealed that DTT supplementation resulted in more than 2 fold improvement of soluble accumulation of RpoB-lysC (see figure 1)

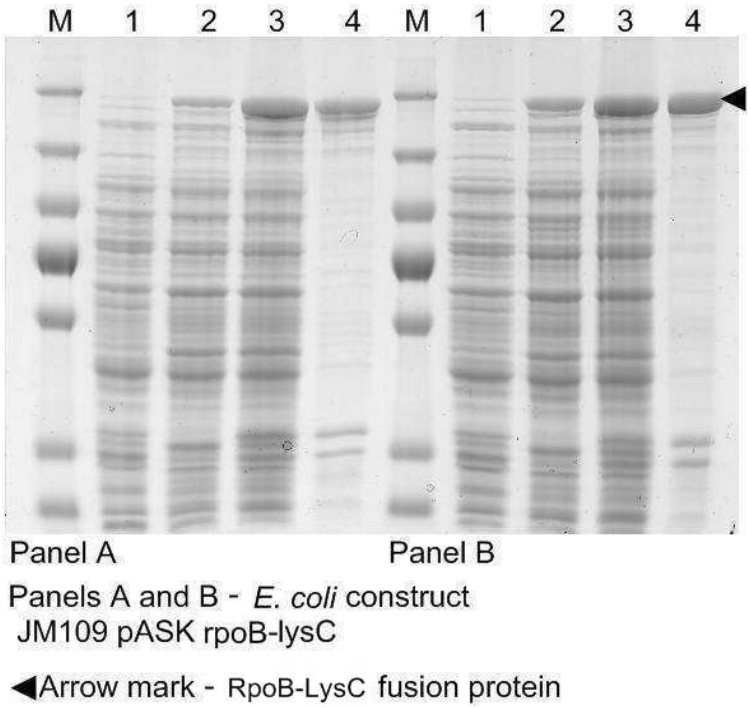

Figure 1. Panels A and B: 8 % SDS - PAGE gel images of normalised to equal amounts protein fractions of production construct: *E. coli* JM109 pASK RpoB-LysC after 4 hours of batch RpoB-LysC production at 30 °C. Panels are representing SDS - PAGE gel images with protein fractions after RpoB-LysC production with no DTT in the medium (Panel A) and with 15 mM of DTT (panel B), respectively. Numbered SDS-PAGE gel lanes represent: 1 - total protein fraction 10 min before induction, 2 - soluble protein fraction, 3 - total protein fraction and 4 – insoluble protein fraction (all 4 h after induction). Protein size marker: PageRuler™ Protein Ladder Plus (Thermofisher scientific).

3.13.2 The DTT mediated folding improvement of thioredoxin and light chain enterokinasefusion protein (TrxA-EK)

The fusion protein comprising thioredoxin A from *E. coli* and light chain enterokinase (TrxA-EK) from bovine contains 11 cysteins. The TrxA-EK is highly aggregating in the T7 RNA polymerase based construct - BL21 pET32a when synthesis is performed at 22℃ (figure 2). The synthesis approach based on medium supplementation with DTT was applied for facilitation of TrxA-EK soluble accumulation in *E.*

coli BL21. The batch production without medium supplementation was included as control.

Inoculums for batch protein production in shake flasks were prepared by overnight batch cultivation of BL21 pET32a TrxA-EK construct in 500 mL shake flaks with 50 ml of LB medium. For protein production 5 % of the corresponding inoculum culture was transferred to fresh LB medium to the final volume of 500 mL in 2 L baffled Erlenmeyer shake flasks. Cultures were cultivated at 37 ℃ and 180 r pm until reached the induction point, corresponding to a cell density of OD_{600}= 0.7 ± 0.05 ($\mu \approx 0.5\ h^{-1}$). Induction was performed by addition of 1 M IPTG to the final concentration of 0.1 mM. DTT was added to the cultivation medium at the TrxA-EK synthesis induction point as dry powder to achieve needed concentration of 15 mM. Cytoplasmic expression was performed for 4 h at 22 ℃ at a shak ing rate of 180 rpm.

After synthesis the harvested cell samples were resuspended in lysis buffer with the 1:10 biomass to buffer ratio in ice cold lysis buffer (50 mM Tris-H_3PO_4 pH 8.0, 0.1 % Triton X-100, 2 mM EDTA, 1 mM PMSF, 2 mM DTT and 0.1 mg mL^{-1} lysozyme). After 30 min of cell lysis the cells were disrupted by sonication for 60 sec (Vibra cell™, Sonic and Materials Inc., 6 mm diameter probe tip) at 4℃. Soluble and insoluble protein fractions were separated by centrifugation for 30 min, at max g and 4℃. After centrifugation the ins oluble protein pellet was additionally washed and resuspended in the original volume of lysis buffer without lysozyme. Samples for SDS-PAGE separation were prepared as follows: 20 µL of protein sample (total soluble, insoluble, protein suspensions), 25 µL of 4×SDS-PAGE loading buffer (Thermofisher scientific) 5 µL of 20×DTT (Thermofisher scientific) and 50 µL of deionised water to obtain a final sample volume of 100 µL. Samples were heated for 15 min at 95^0℃. 10 µL of sample was applied to each lane of a 10 % SDS-PAGE gel. Finally, the differences in the amounts of Trx-EK in soluble fractions after test productions with and without DTT in the medium were evaluated from scanned SDS-PAGE gel images by image treatment with TotalLab software.

The results analysis showed that DTT significantly improved TrxA-EK soluble accumulation, despite 4-5 fold decreased total protein accumulation level.

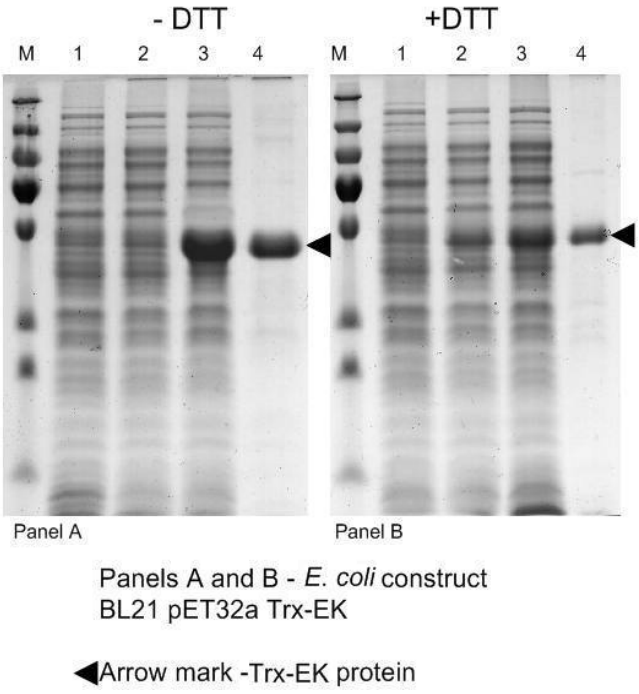

Figure 2. Panels A and B: 10 % SDS - PAGE gel images of normalised to equal amounts protein fractions of production construct *E. coli* BL21 pET32a-Trx-EK after 4 hours of batch Trx-EK production at 22 °C**.** Panel A represents SDS - PAGE gel image with protein fractions after Trx-EK production with no DTT in the medium and panel B, - with 15 mM of DTT respectively. Numbered SDS-PAGE gel lanes represents: 1 - total protein fraction 10 min before induction, 2 - soluble protein fraction, 3 - total protein fraction and 4 – insoluble protein fraction after 4 h of Trx-EK synthesis induction. Protein size marker: PageRuler™ Protein Ladder Plus (Thermofisher scientific (Fermentas)).

3.13.3 DTT mediated folding improvement of pancreatic bovine DNaseI

The DNaseI (31 kDa) from bovine pancreas contains 4 cysteines, which forms two intermolecular disulfide bonds (Price et al. 1969).

Surprisingly, the DNase I batch production in K12 HMS174 T7 RNA polymerase with DTT addition to the cultivation medium resulted in 4 fold enzyme activity improvement per cell and 40 % improvement of total DNaseI accumulation (figures 3 and 4).

The inoculums for batch protein production in the shake flasks were prepared by overnight batch cultivation of the selected clone in 500 mL shake flaks with 50 ml of LB medium. For protein production 5 %

of the inoculum culture was transferred to 500 mL of fresh semi-synthetic medium with following composition: 4.1 g L^{-1} KH_2PO_4, 3.2g /L K_2HPO_4 (pH 7.0 adjusted), 5g/L NaCl, 5 g/L yeast extract, 10g/L tryptone and 5 g/L glycerol. Cultures were cultivated at 37℃ and 180 rpm until they reached the induction point corresponding to cell density of OD_{600}= 0.7 ± 0.05 (μ ≈ 0.3 h^{-1}). Induction was performed by addition of 1 M IPTG to the final concentration of 0.1 mM. The DTT was added to the cultivation medium at the DNaseI synthesis induction point as dry powder to achieve the final concentration of 15 mM. Cytoplasmic propagation was carried for 4 h at 37℃ at a shaking rate of 180 rpm.

After synthesis the harvested cell samples were resuspended in lysis buffer with the 1:10 biomass to buffer ratio in ice cold lysis buffer (50 mM Tris-H_3PO_4 pH 8.0, 0.1 % Triton X-100, 1 mM $CaCl_2$ and 0.1 mg mL^{-1} lysozyme). After 30 min of lysis at 4 ℃, the biomass was disrupted by sonication for 60 sec (Vibra cell™, Sonic and Materials Inc., 6 mm diameter probe tip) at 4 ℃. Soluble and insoluble protein fractions were separated by centrifugation at max g and 4 ℃ for 30 min. Total protein fraction is represented by cellular debris suspension (crude extract) before centrifugation.

For DNaseI activity assay cell crude extracts after recombinant production were diluted in lysis buffer (without lysozyme) as follows: 50, 250, 1000, 2500, 5000, 10000, 15000, 20000 and 25000 times. The commercial DnaseI (Thermofisher scientific, #EN0521) was diluted in lysis buffer as follows: 50, 250, 500, 1000, 1500, 2000, 2500, 3500 and 5000 times. The reaction was performed in 30 μL mixture with composition of 1 μg of pUC57 DNA, DnaseI 10x reaction buffer (100 mM Tris-HCl (pH7.5 at 25 ℃), 25 mM $MgCl_2$, $CaCl_2$ 1 mM), 5 units (0.1 μL) of RNaseA (Thermofisher scientific, #EN0531), 1 μL of diluted sample (or commercial enzyme) and required amount of nuclease free water. The reaction was performed for 10 min at 37 ℃ and stopped by heating mixture at 70 ℃ for 10 min, after supplementation with SDS containing loading dye (Thermofisher scientific, #R1151) and Na_2EDTA (final concentration of 5 mM).

Finally, 15 µL of inactivated reaction mixture was applied on 1 % agarose gel for DNA electrophoresis.

Samples for SDS-PAGE separation were prepared as follows: 20 µL of protein sample (total soluble, insoluble, protein suspensions), 25 µL of 4×SDS-PAGE loading buffer (Thermofisher scientific), 5 µL of 20×DTT (Thermofisher scientific) and 50 µL of deionised water to obtain a final sample volume of 100 µL. Samples were heated for 15 min at 95^{0}℃. 10 µL of sample was applied to each lane of a 1 0 % SDS-PAGE gel. Differences in amounts of DNaseI in soluble fractions after productions with and without DTT in the medium were evaluated from scanned SDS-PAGE gel images by image treatment with TotalLab software.

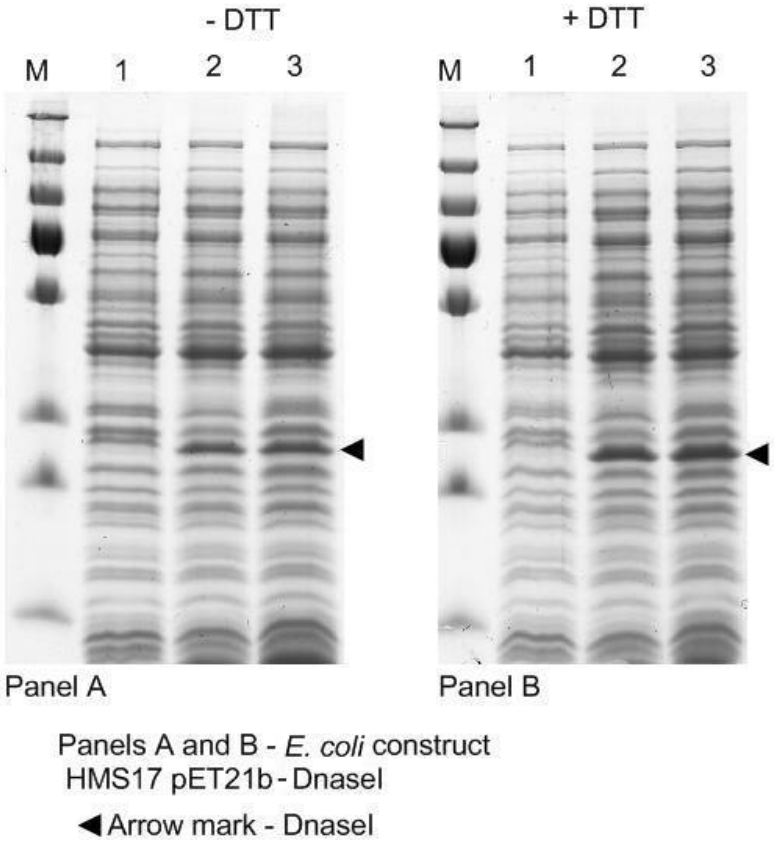

Figure 3. Panels A and B: SDS - PAGE gel images of normalised to equal amounts protein fractions of *E. coli* production construct HMS174 pET21-DnaseI after 4 hours of batch DNaseI production at 37 °C**.** Panel A represents 10 % SDS - PAGE gel image with protein fractions after DNaseI production with no DTT in the medium and panel B, - with 15 mM DTT respectively. Numbered SDS-PAGE gel lanes represents: 1 - total protein fraction 10 min before induction, 2 - soluble protein fraction and 3 - total protein fraction 4 h afterDnaseI synthesis induction. Protein size marker: PageRuler™ Protein Ladder Plus (Thermofisher scientific).

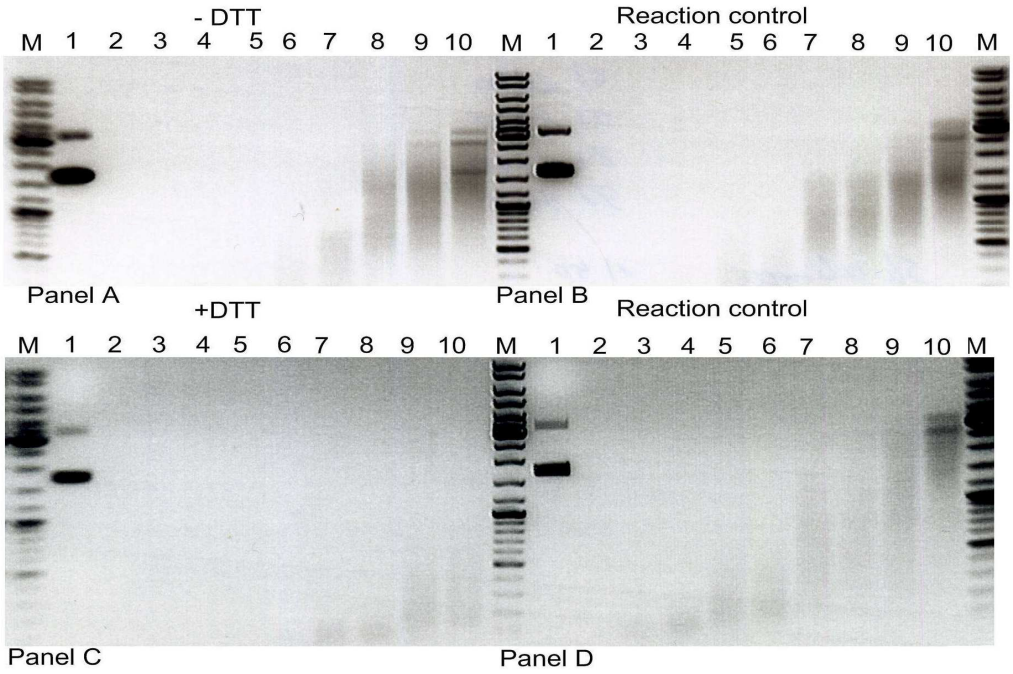

Figure 4. Panels A-D: images of 1 % agarose gel after DNA electrophoresis, with samples generated after soluble protein extracts (Panels A and C) and reference DNaseI (Panels B and D) incubation with 1 µg of pUC57 DNA in DNaseI reaction mixture. In panels A and C, gel lanes are representing samples from reaction mixtures with different dilutions (lane 2: ×50, lane 3: ×250, lane 4: ×1000, lane 5: ×2500, lane 6: ×5000, lane 7: ×10000, lane 8: ×15000, lane 9 : ×20000 and lane 10: ×25000 times) of soluble protein fraction after recombinant production of DNaseI in HMS174 pET21 DNaseI construct at 37℃ in the cultiv ation medium without DTT (Panel A) and with 15 mM of DTT (Panel C). In panels B and D gel lanes are representing samples from reaction mixtures with different dilutions (lane 2: ×50, lane 3: ×250, lane 4: ×500, lane 5: ×1000, lane 6: ×1500, lane 7: ×2000, lane 8: ×2500, lane 9: ×3500 and lane 10: ×5000 times) of commercial DNase I. In all gels, lane 1 is representing pUC57 DNA without treatment with DNaseI samples.

3.13.4 DTT-mediated folding improvement of SssI methyltransferase from Spiroplasma sp.

The CpG methyltransferase - SssI from *Spiroplasma sp*, contains 2 reduced cystein residues, when molecule is the native conformation. The Cys141 residue, present in the enzyme catalytic centre is highly important for enzyme catalytic features (Rathert et al. 2007). The enzyme is highly aggregating during recombinant production in the *E. coli* cells (see expression results bellow). In order to facilitate SssI folding in *E. coli* strain production construct - ER2566 pACY184-TT-SssI, the batch production approach based on medium supplementation with DTT was applied. The addition of DTT resulted in improvement of active SssI accumulation per cell (figure 5 and figure 6).

The inoculums for batch protein production in the shake flasks were prepared by overnight batch cultivation of the selected clones in 500 mL shake flaks with 50 ml of LB medium. For protein production 5 % of the corresponding inoculum culture was transferred to fresh LB medium to the final volume of 500 mL in 2 L baffled Erlenmeyer shake flasks. Cultures were cultivated at 37 ℃ and 180 rpm until they reached the induction point corresponding to cell density of OD_{600}= 0.7 ± 0.05 ($\mu \approx 0.5\ h^{-1}$). Induction was performed by addition of 1 M IPTG to the final concentration of 0.1 mM. The DTT was added to the cultivation medium at the SssI synthesis induction point as dry powder to achieve final concentration of 15 mM. Cytoplasmic expression was carried out for 4 h at 30 ℃ at the shaking rate of 180 rpm.

After synthesis the harvested cell samples were resuspended in lysis buffer with the 1:5, biomass to buffer ratio in ice cold lysis buffer (10 mM K-phosphate buffer pH 7.4, 10 % glycerol, 200 mM NaCl, 1mM EDTA, 1 mM DTT, 0.1 % Tween-20 and 0.1 mg mL^{-1} lysozyme). After 30 min of lysis at $+4^{0}$℃ the biomass was disrupted by sonication for 60 sec (Vibra cell™, Sonic and Materials Inc., 6 mm diameter probe tip) at 4 ℃. Soluble and insoluble protein fractions were separated by centrifugation for 30 min, at max g, 4 ℃. Total protein fraction is represented by cellular debris suspension (crude extract) before centrifugation. After centrifugation the insoluble protein pellet was additionally washed and resuspended in the original volume of lysis buffer without lysozyme.

For SssI methyltransferase activity assay, crude cell extracts were diluted (5 times) with buffer: 20 mM K-phosphate buffer (pH 7.4), 7 mM 2-mercaptoethanol, 0.2 mg/ml BSA, 1mM Na_2EDTA, 200 mM KCl and 10 % glycerol. The SssI methyltransferase activity was evaluated by performing λ-phage DNA methylation reaction in the mixture with the following components: 0.5, 1 2 and 3 µL of diluted crude extracts, 1 µg λ-phage DNA, 50 µL Tango Buffer (Thermofisher scientific), 100 µM S-adenosylmethionine (SAM). The reaction was performed at 37 ℃ for 1 h. The reaction was stopped by 20 min heating at 65 ℃. After methylation reaction the λ-phage DNA was incubated with 1 µL (1 unit) of HpaII restriction enzyme (Thermofisher scientific, #ER0511),

for 30 min at 37 ℃. The reaction was stopped by heating at 65 ℃ for 10 minutes. Afterwards mixture was supplemented with SDS containing loading dye (Thermofisher scientific, #R1151) and Na_2EDTA (final concentration of 5 mM). 15 µL of inactivated reaction mixture was applied on 1 % agarose gel for DNA electrophoresis.

Samples for SDS-PAGE separation were prepared as follows: 20 µL of protein sample (total soluble, insoluble, protein suspensions), 25 µL of 4×SDS-PAGE loading buffer (Thermofisher scientific), 5 µL of 20×DTT (Thermofisher scientific) and 50 µL of deionised water to obtain a final sample volume of 100 µL. Samples were heated for 15 min at 95 ℃. 10 µL of sample was applied to each lane of a 10 % SDS-PAGE gel.

Differences in the amounts of SssI in soluble protein fractions after test productions with and without DTT in the medium were evaluated from scanned SDS-PAGE gel images by image treatment with TotalLab software.

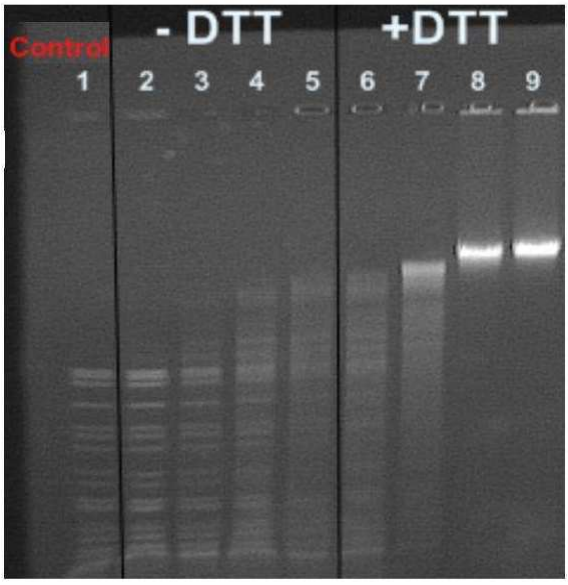

Figure 5. Image of 1 % agarose gel after DNA electrophoresis, with samples generated from soluble protein extracts incubation with 1 µg of λ-phage DNA in SssI methyltransferase reaction mixture, followed by digestion with HpaII. Gel lane 1, "control" **-** λphage DNA without methylation, after 30 min incubation with HpaII at 37℃**.** Gel lanes 2-5, represents samples from methylation/restriction reactions with 0.5, 1, 2 and 3µL of crude extracts, generated after SssI synthesis without DTT in the cultivation medium. Gel lanes 6-9, represents samples from methylation/restriction reactions with 0.5, 1, 2 and 3µL of crude extracts, generated after SssI synthesis with DTT in the cultivation medium.

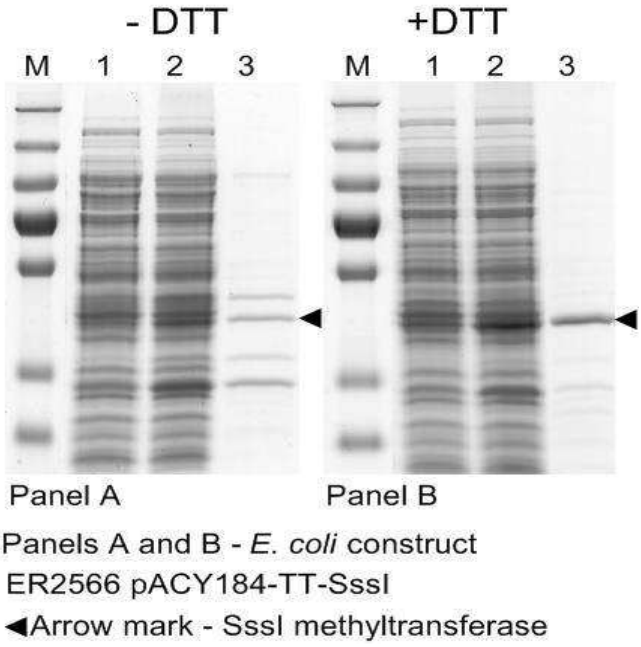

Figure 6. Panels A and B: 10 % SDS - PAGE gel images of normalised to equal amounts protein fractions, of *E. coli*pACY184-TT-SssI production construct after 4 hours of batch SssI production at 30 °C Panels are representing SDS - PAGE gel images with protein fractions after SssI production with no DTT in the medium (Panel A) and with 15 mM DTT (panel B), respectively**.** Numbered lanes in the SDS-PAGE gels are representing: 1 - soluble, 2- total and 3- insoluble protein fractions, 4 h after SssI synthesis induction. Protein size marker: PageRuler™ Protein Ladder Plus (Thermofisher scientific).All cultures were initially started at 37 ℃, induced with 0.1 mM of IPTG. The SssI synthesis was performed at 30 ℃.

4 Discussion

4.1 The novel straightforward process development concept

4.1.1 Microscale screening under substrate-limited mode

In work presented here, the novel biocatalysis based substrate auto-delivery technique known as Enbase® (Peralla et al. 2008) was employed for the development and scale-up of several target RI protein production processes under fed-batch growth (papers I-III).

Initially, the RI propagation was attempted in the cytoplasmic and periplasmic compartments of *E. coli* cells. Therefore, two complementary, systematic cytoplasmic and periplasmic expression vector libraries and the luminescence based folding stress reporting systems (see paper I and paper II) were utilized to screen for the optimal cytoplasmic and periplasmic RI propagation constructs. The Enbase® cultivation technique allowed to perform the micro-scale high-throughput screening for the desired constructs in the substrate-limiting mode, i.e. at the final-scale-like growth conditions (paper I and paper II).

Despite slightly different techniques that were used to prepare the inoculum cultures, the cytoplasmic and periplasmic production constructs exhibited similar growth patterns during the pre-induction growth phase and high capacity for the recombinant protein production (paper I figure 2 and paper II figure 1). After RI synthesis induction, however, the growth patterns of the cytoplasmic and periplasmic constructs diverged. After the addition of IPTG, all cytoplasmic constructs continued to grow independently from the vector elements and the post-induction temperature (paper I, figure 2). Interestingly, only the periplasmic constructs possessing weaker expression elements (promoters *pC-, pCU-,* RBS: *-lac, - var*) continued to grow, while the growth of the periplasmic constructs harbouring strong expression elements was completely arrested

(paper II, figure 2). It was assumed, that the growth inhibition of the periplasmic constructs could occur due to the withdrawal of cellular resources by a strong RI expression at the substrate-limiting mode and/or toxic effects caused by a strong RI aggregation in the periplasmic space of the cell (paper II, figure 1) (Hunke et al. 2003).

In summary, the Enbase® micro-scale cultivation technique showed a high degree of robustness, thus, reproducible growth patterns and consistent expression data at all tested production temperatures were obtained.

The screening approach presented in this work (paper I) was already successfully utilized by the other authors. For example, 96 human proteins were successfully synthesized in 96-well format microwell (150 µL) and 24-well format deep-well plates (3 mL) (Tegel et al. 2011). All produced proteins after purification with IMAC chromatography were evaluated by MALDI-TOF-MS technique. The collected data revealed highly similar accumulation patterns for all proteins, independently from the production scale. The MS data showed that all produced proteins maintained qualitative parameters, coinciding even for 95 % of them (Tegel et al. 2011).

Worth mentioning, that there is a number of alternative, but more sophisticated miniaturized cultivation platforms. For example, miniaturized mechanical pump-based bioreactors also allow to evaluate the construct behaviour under the substrate-limiting cultivation conditions. (Scheidle et al. 2010, Hortsch et al. 2010, Fernandes et al. 2011).

4.1.2 The evaluation of RI aggregation by cytoplasmic and periplasmic folding stress reporter systems

The successful application of the cytoplasmic folding stress reporter system based on *pibpfxsT7lucA* was previously reported with the folding-defective variant of the maltose-binding protein and other partially insoluble heterologous model proteins (Kraft et al. 2007). In addition, the periplasmic folding stress reporter system *plt1* allowed to identify the constructs for the soluble propagation of the human 11β-

hydroxysteroid dehydrogenase type 2 and scFv-miniantibody phosphatase in the periplasmic space of *E. coli* RV308 (Kraft et al. 2007).

However, in this work only the cytoplasmic reporter system allowed to identify the constructs accumulating tagged MBP-RI as a soluble protein, by generating the luminescence signals by 2-3 fold lower, compared to the clones accumulating aggregating RI fusions (paper I, figure 3).

The periplasmic aggregation reporter *plt1* did not suit for the assessment of the RI periplasmic accumulation. Unexpectedly, the periplasmic propagation systems produced much lower luminescence signals in the constructs harbouring strong expression elements, in which the target protein accumulated in the insoluble protein fraction (paper II, figure 1). It was assumed that a very strong RI expression at the substrate-limiting mode resulted in the withdrawal of cellular resources, thus, the propagation and/or folding of the recombinant luciferase was restricted. In addition, in the original work the *plt1* system was demonstrated under completely different cultivation and synthesis conditions, i.e. different composition of cultivation medium and stirred-tank bioreactor instead of microwell plates (Kraft et al 2007). Therefore, it would be beneficial before the screening trails to evaluate the misfolding monitoring systems with known aggregate-prone model proteins at the cultivation conditions, which will be present during the screening for the desired construct.

4.1.3 The conditional screening in the fed-batch shaken flasks

The classical recombinant process development and the scale-up typically starts from the recombinant production construct optimization, usually in the shaken flasks and continues in the larger scale stirred-tank bioreactor. This approach cannot always guaranty a successful outcome, due to the significant differences in the culturing and synthesis conditions in different cultivation vessels/scales (Fernandes et al. 2011).

In this work we found a simple approach for screening for the best fed-batch pre-induction growth conditions to propagate the target protein directly in the shaken flasks, thus, a number of sophisticated procedures in stirred-tank bioreactor was eliminated. The presented conditional screening approach is based on the previously discussed Enbase® cultivation technique, which is also suitable for the fed-batch cultivations in the shake flasks.

The first group of fed-batch synthesis experiments, which gave the basics for the conditional screening approach, was performed with the cytoplasmic production construct RV308/pibpfxsT7lucA/pCTUT7MBP-RI. This construct was considered as the most suitable for further His_6-MBP-RI optimisation trails (paper I, “Results” section).

Prior to the RI production trials in the shaken flaks under Enbase® generated substrate-limited mode, the number of fed-batch cultivation experiments with the selected construct without the target protein synthesis were performed. (paper I, “Materials and Methods” section). The cultivation results showed that the growth rate of RV308/pibpfxsT7lucA/pCTUT7MBP-RI constructs was gradually decreasing in relation to the increasing cell density and increasing substrate limitation (paper I, figure 5). The synthesis induction at the growth rates of 0.33, 0.22 and 0.11 h^{-1} allowed to evaluate the effect of the specific growth rate on the His_6-MBP-RI accumulation and/or folding (paper I, figure 5). The synthesis results revealed that the substrate limited mode corresponding to μ of 0.22 h^{-1} was the most favourable for the His_6-MBP-RI cytoplasmic propagation. However, the cellular activity for the recombinant production could not be maintained under the substrate-limited growth conditions, corresponding to μ of 0.1 h^{-1}, thus, the amount of obtained His_6-MBP-RI was significantly lower (paper I, figure 6). These findings are in good agreement with the previous reports, which showed that the optimal growth rate for the induction of the recombinant protein synthesis is in the range of 0.15 - 0.3 h^{-1} (Bentley et al. 1990, Flickinger et al. 1990). In addition, specific growth rate of the pre-induction phase determines the level of the target protein accumulation (Curless et al. 1990), folding quality (Hoffmannet al.

2004) and the quality of the posttranslational modifications (Curless et al. 1994, Ihssen J et al. 2011). Therefore, specific growth rate of the pre-induction phase is an important parameter, which should be considered and evaluated during the bioprocess development

Finally, in order to evaluate the His_6-MBP-RI accumulation at the maximal substrate inflow, the set of simple batch production trails were performed in the same MSM glucose medium, MSM medium with complex additives and in classical LB medium (paper I, “Materials and Methods section”). The His_6-MBP-RI batch synthesis results revealed that target protein accumulation level depends on the growth mode and His_6-MBP-RI aggregation is highly depended on the medium composition, i.e. complex medium compounds (paper I, Figure 6). It was assumed that during batch synthesis the complex medium compounds enhanced the overflow metabolism, stipulated imbalanced rapid cell growth and, thus, created conditions promoting the aggregation of target protein (Boström et al. 2005, Bäcklund et al. 2011). Nevertheless, the complex medium compounds are beneficial for the recombinant protein accumulation and folding when the substrate inflow is well balanced in relation to the cellular respiration (Krause et al. 2010).

It is worth to mention that the medium composition/growth mode had also a significant impact on the folding of other model proteins, for example alcohol dehydrogenise (ADH) (from *Lactobacillus* brevis) and formate dehydrogenase (FDH) (from Candida boidinii), in *E. coli* based constructs (Hortsch et al. 2011).

4.1.4 The verification of fed-batch scale-up approach in the stirred-tank bioreactor

The consistency of the results derived in the shaken flasks was assessed by carrying a set of His_6-MBP-RI fed-batch production trials in the stirred-tank bioreactor. The mechanical pump-based feeding system was tuned to maintain specific growth rate μ of 0.22 h^{-1}, which was considered as optimal for the His_6-MBP-RI synthesis induction (paper I, figure 8). Despite that the His_6-MBP-RI synthesis was

induced at the different cell densities, both fed-batch production processes yielded highly similar accumulation patterns of the total and soluble His_6-MBP-RI. In addition, the His_6-MBP-RI accumulation in stirred-tank bioreactor was determined as highly similar to the results from the analogical fed-batch production trails in the shaken flasks (paper I, figure 9). Therefore, it was considered that the Enbase® fed-batch substrate-limited cultivation technique creates highly similar growth conditions to ones found in the aerated stirred-tank bioreactor.

Contrary, the His_6-MBP-RI accumulation patterns, obtained after the batch synthesis in stirred-tank bioreactor, were completely different from the analogical batch production results obtained in the shaking flasks (paper I, figure 7 and figure 9). Once again, this data showed that the straightforward production process transfer/scale-up from one batch to the other might result in unpredictable target protein accumulation.

In summary, in the first part of the work the significance of cultivation parameters for the recombinant construct productivity was clearly demonstrated, as well as the unpredictable recombinant construct behaviour after the process transfer from the batch shaking flasks to the stirred-tank batch-type bioreactor. Therefore in the following stages of research, during the process scale-up, the optimal production conditions for RI folding were verified by using the above descried fed-batch development approach.

4.2 Factors influencing RI folding in *E. coli* based production constructs

4.2.1 The MBP N terminus tag - an effective RI solubility enhancer

The results from the fed-batch production trails in the microwell plates (discussed in the section above) showed that the RI accumulation in soluble fraction highly depends on the N-terminal MBP tag. Surprisingly, the transcription and/or translation elements regulating the expression rate had a minor effect on the RI

cytoplasmic accumulation in the soluble protein fraction in all expression constructs (paper I, figure 2). Recently, highly comparable results of murine RI (mRI) production in *E. coli* were presented by Guo and co-workers. The authors found that the highest amount of soluble mRI in T7 RNA production construct was also dependent on the N-terminalfusion with MBP (Guo et al. 2011). Therefore, it was assumed, that the exclusive effect of the N terminal MBP tag on RI soluble accumulation was observed due to MBP chaperon-like action on RI folding (Bach et al. 2001). However, in our case the N-terminal MBP fusion decreased the ability of RI to inhibit RnaseA by more than 9 fold (unpublished data). Maybe MBP blocks the access to the interior part of RI molecule, thus, the inhibitor cannot interact with the RNaseA.

4.2.2 Redox mediated RI folding in K12 RV308 *E. coli* construct

The production trails using various amounts of GSH and DTT were performed with the constructs harbouring all four leader peptides, and genetic elements stipulating weak and strong expression. In addition, in order to eliminate the possible RI oxidation by the periplasmic oxido-reductases, the parallel experiments were carried out with the analogical set of constructs deficient in the *dsbA* gene. The medium supplementation with reducing agents showed that only DTT is an efficient RI folding modulator, when the production is monitored in the *dsbA* positive and deficient constructs with weak expression elements (paper II, figure 4).

The examination of insoluble protein fraction after RI periplasmic propagation under reducing conditions showed that not all of the processed RI pool remained in the soluble state after the cleavage of leader peptide. Approximately 50 % of the processed target protein aggregated after the cleavage of leader peptide in all *dsbA*$^-$ and isogenic constructs (paper II, figures 2 and figure 3). Therefore, it was assumed that besides the reducing environment RI requires stabilization and/or folding assistance to eliminate or reduce the hydrophobic aggregation. Worth mentioning, that similar patterns of

periplasmic aggregation of recombinant protein were also observed previously by other authors (Bowden et al. 1994, Arié et al. 2006, Ario de Marco et al. 2009).

Interestingly, there was no clear relation seen between the utilized secretion leader peptides and RI periplasmic accumulation and/or folding efficiency. However, the insignificant advantage of MalE and PelB leader peptides for the RI periplasmic translocation was indicated in *dsbA* deficient and isogenic construct, respectively (paper II, figure 4). Finally, the effect of elimination of periplasmic oxireductase *dsbA* confirmed our assumption that the RI activity could be decreased by periplasmic oxireductases. The RI periplasmic activities per cell were obtained higher in *dsbA* deficient constructs, compared to the results obtained in analogical isogenic constructs after the production with higher amount of DTT (paper II, figure 4). On the other hand, the *dsbA* deficient strain showed much higher sensitivity to DTT, thus, exhibited a lower productivity and pure growth, compared to the isogenic constructs. In addition, the *dsbA* deficient constructs were not productive under substrate-limited conditions when medium was supplemented with DTT. Thus, bioreactor production trails were performed only with the intact *dsbA* gene possessing *E. coli* RV308 pCUlac-pelB-RI construct.

The analysis of cellular fractions after the RI periplasmic production under reducing conditions showed that besides improved periplasmic accumulation, the addition of DTT resulted in the increase insoluble accumulation of the unprocessed RI, and the total RI activity per cells (paper II, “Results” section). Therefore, it was assumed that in addition to the periplasmic compartment DTT is also positively acting on RI folding in cell cytoplasm. The same batch production protocol was applied for the RI cytoplasmic propagation in the *E. coli* K-12 strain RV308. The experimental results revealed that RI folding in the cytoplasmic space of *E. coli* is highly dependent on the synthesis temperature and DTT concentration in the cultivation medium (paper II, Figure 5).

In summary, it was indicated that the genetic elements stipulating weak expression in combination with low production temperature are

the key factors for the efficient redox-depended periplasmic and cytoplasmic RI propagation in *E. coli* K12 constructs. The reducing agents had no effective in strong expression elements possessing constructs (paper II). No doubt, the optimal conditions for the RI propagation are highly stressful for the cells (paper IV). The comparative proteome analysis revealed an up-regulation of the σ^{32}-related proteases Lon, HslVU and ClpB, (paper IV), which might have a negative impact on the recombinant protein yield (Rozkov et al. 2004).

4.2.3 Factors limiting RI folding in *E. coli* cells

The deeper understanding about the bottlenecks in cellular folding machinery, which are limiting the soluble RI propagation in *E. coli* cells, was gained after a series of expressions trails with native sequence possessing RI in T7 RNA polymerase system - ER2566/ pET21b (paper III). The first set of the RI propagation experiments showed that in T7 RNA polymerase system stipulating strong expression the RI was highly aggregating, even when the production was carried out at low temperature under highly reducing conditions (paper III, figure 1 and figure 2). In order to evaluate the folding bottlenecks, which limit soluble accumulation of RI, the RI was propagated at the different temperatures with co-expression of the main *E. coli* chaperon groups: DnaK/J/GrpE (Sharma et al. 2010) and GroEL/ES (Masters et al. 2009). Interestingly, the DnaK/J/GrpE chaperon group had only a minor positive effect on RI solubility, but did not improve the activity (paper III, figures 1 and 2). This result was quite unexpected, because according to a numerous literature reports, the co-expression of the DnaK/J/GrpE chaperon system is a powerful tool for the prevention of target protein aggregation (Kolaj et al. 2009). On the other hand, it is possible that RI doesn't require the assistance from the DnaK/J/GrpE chaperon system, which at the early stages of folding pathway stabilizes nascent peptides by interacting with the hydrophobic patches of the client protein (Young et al. 2004, Choi et al. 2011).

In contrary, the co-expression of the GroEL/ES chaperon systems catalyzing the folding resulted in a higher RI activity and improved solubility of the product (paper III, figure 1 and figure 2), indicating that RI requires folding aid in the late folding stages. The positive effect of GroEL/ES on the RI activity also indicates that RI (49 kDa) was encapsulated in the GroEL/ES complex cavity, where it was re/folded and/or stabilized (Masters et al. 2009, Platas et al. 2011).

In addition, the series of RI production trails with the GroEL/ES co-expression showed that this chaperonin complex exhibited a certain capacity for the RI folding. The most favourable synthesis conditions - the low temperature, reducing conditions and full induction of chaperon synthesis, resulted in up to 25-27 mg $gCDW^{-1}$ in the soluble protein fraction (paper III, figure 1 and figure 2). To explain these interesting findings two assumptions were made. Firstly, it is possible that the high co-expression level of GroEL and GroES subunits yields a pool of incorrectly assembled, non-functional GroEL/ES chaperonin systems. Secondly, even if all chaperon barrels are correctly assembled it is barely possible that the cells are capable to provide enough ATP to r maintain all the synthesized chaperonin units in functional mode.

4.2.4 The “complex” RI folding approach

The effect of redox situation on RI folding during chaperon co-expression was verified after a set of RI production trails based on the cytoplasmic redox modifications with DTT. In order reduce the possible physiological stress elucidated by strong synthesis induction and DTT toxicity the reducing agent was added at the time of RI induction and 2 hours after (paper III, “Materials and Methods” section). Interestingly, DTT improved the RI activity and solubility per cell only in the construct harbouring GroEL/ES chaperon complex. The combination of DTT supplementation and co-expression of DnaK/J/GrpE further facilitated soluble RI accumulation, but not the activity (paper III, figure 1 and figure 2).

The explanation for this phenomenon came after careful SDS-PAGE gel inspection, where it was noticed that the band representing DnaJ is present in the insoluble protein fraction together with the aggregated RI even after production at low temperature (paper III, additional figure 2). Therefore, it is possible that the low RI activity was observed due to tight DnaJ interaction with hydrophobic patches of RI molecule, thus, RI interaction with the RNaseA was very limited (during the activity evaluation *in vitro*). In addition, in previous reports was demonstrated that the solubility improvement mediated by the DnaK/J/GrpE co-expression is not always accompanied by the increase of target protein biological activity (Ikura et al. 2002, Martínez-Alonso et al. 2007)

The DTT addition at the RI synthesis induction point had a slightly better effect on the RI activity in the ER2566 pET21bRI pGro7 construct, compared to the delayed DTT supplementation. However, delayed DTT addition resulted in a higher cellular productivity and higher RI accumulation into the soluble fraction, thus, total RI activity per cell was improved. As it was expected, the DTT supplementation resulted in a huge loss of the RI total amount in all T7 promoter based constructs (paper III, figure 2). Interestingly, the mentioned effect was only observed when DTT was added at the RI synthesis induction point and the synthesis was continued at high temperature. The derived results showed that DTT supplementation at the synthesis induction point makes cells physiologically very vulnerable, thus, the strong induction and high synthesis rate significantly diminished the yield of the target protein. This conclusion is supported by several literature reports, here authors clearly indicate that a strong induction creates so called metabolic burden (Glick et al. 1998) and has a negative impact on cellular physiology and productivity (Dong et al. 1995, Neubauer et al. 2003, Lin et al. 2004).

In addition, a comparative proteome analysis performed aside from this work (see manuscript (paper IV)) revealed that RI production in the construct possessing weak expression elements at low temperature and reducing conditions resulted in a significant induction of stress related heat-shock, oxidative-stress and proteolytic activity

possessing proteins (paper IV). Thus, we consider that the stress response in T7 RNA construct at the higher production temperature was induced even stronger, especially, when DTT was added at the time of RI synthesis induction.

4.2.5 The redox dependent RI folding under substrate limited conditions in the shaking flasks

The fed-batch synthesis trails in the shaking flasks allowed to evaluate the behaviour of the periplasmic and cytoplasmic RI production constructs during RI production under reducing conditions and low temperature. The fed-batch production trials showed that due to medium supplementation with DTT *dsbA* negative RV308 periplasmic production construct was not productive at the substrate-limited growth conditions. In addition, the cytoplasmic and periplasmic *dsbA*$^{+}$ constructs produced similar patterns of total and soluble RI accumulation under substrate-limited mode, compared to the analogical batch trails. Therefore, it was assumed that DTT has a similar effect on RI folding, independent, from substrate availability (paper II, figure 6).

The same scale-up approach was applied for evaluation of RI accumulation in the ER2566 pET21bRI pGro7 construct under substrate-limited growth with co-expression of GroEL/ES under reducing conditions. Differently from the above described fed-batch trails, the ER2566 pET21bRI pGro7 construct gave completely different RI accumulation pattern compared to the analogical batch production process (paper III, figure 1 and figure 3). The addition of DTT at the same time as IPTG drastically decreased the RI accumulation level, even when production was carried out at the low temperature (results not shown).

Interestingly, the pre-induction specific growth rate had a significant effect on the RI activity and on accumulation level. The RI synthesis levels in T7/GroEL/ES construct were similarly decreased after induction at μ of 0.22 h^{-1} and μ of 0.1 h^{-1}. In addition, the obtained amounts of soluble RI were also highly similar to the analogical batch

production process. However, the RI production under restricted substrate supply corresponding to μ of 0.1 h^{-1} resulted in a much lower RI activity per biomass, compared to the process where synthesis induction was at μ of 0.22 h^{-1} (paper III, figures 2 and figure 3).

It was assumed that the loss of productivity in the T7 system was caused by the huge metabolic load under restricted substrate supply (Andersson et al. 1996, Notley et al. 1996, Teich et al. 1999). The withdrawal of energy, which is required for RI folding, was drained by cells for the maintenance of cellular activities under very stressful conditions - response to addition of toxic DTT, over-expression of heterologous proteins (Gill et al. 1998). Therefore, the exponential feeding profile for stirred bioreactor process was designed to maintain of specific growth rate μ of 0.22 h^{-1} at the pre-induction growth stage.

4.2.6 The redox dependent RI folding and propagation in a stirred-tank bioreactor

The DTT mediated RI folding was also reproduced in stirred-tank bioreactors under substrate-limited conditions. In order to preserve DTT from oxidation, prior to the DTT supplementation the air flow was down regulated to maintain the oxygen concentration in the cultivation medium close to 0 %. The first RI bioreactor production trials in K12 RV308 constructs were quite disappointing. The approach, based on the air flow down-regulation, addition of DTT and RI synthesis induction at the same time point, resulted in strongly diminished cellular productivity (paper II).

This effect could be expected, because oxygen limited conditions are not favourable for the recombinant production due to reduced energy production, medium acidification and intoxication with the intracellular organic side metabolites. In addition, besides the overflow metabolism, the low aeration production conditions could cause an elevate protein aggregation, decrease in cellular productivity and inhibited cellular growth (Neubauer et al. 1995). The recent report showed that accumulation of toxic by-products at the low aeration conditions could be at least partially reduced by using a special

combination of microelements, containing increased concentrations of selenium, nickel and molybdenum (Soini et al. 2008).

The cellular productivity was regained after oxygen down-regulation and medium supplementation with DTT were separated from the RI synthesis induction. This approach allowed to obtain total RI accumulation level in K12 RV308 strain, similar to the levels achieved in the EnBase® shaking flasks. However, despite regained productivity of the cell, the RI cytoplasmic and periplasmic activities not still were much improved (paper II, figure 8). It was suspected that even the low air inflow oxidizes a crucial amount of DTT, which instead of SH group stabilization, starts acting as an oxidizing agent. Our suspicions were confirmed after the analysis of DTT oxidation patterns, which showed that after a single supplementation with DTT more than 60% of DTT was oxidized during 3-hours synthesis course, even when the oxygen level was maintained close to 0% (paper II, figure 9). Another conformation came from the comparative proteome analysis, which revealed, that 3-hours production course under limited aeration conditions and DTT in the cultivation medium resulted in a drastic response of proteins responsive to oxidative stress, which, we believe was caused by oxidized form of DTT (discussed bellow, see paper IV).

By taking these data into account, favourable redox conditions for the cytoplasmic and periplasmic RI folding in the stirred-tank bioreactor were created by introducing the repeated DTT pulsing approach (paper II, “Materials and Methods” section).

As it was expected, the DTT was much less oxidized in the shaking flasks (paper II, figure 9). Therefore repeated DTT pulsing was not required for complete facilitation of the RI folding in the shaking flasks.

The single and repeated DTT pulsing approaches were also used for RI propagation in the T7 RNA polymerase system with GroEL/ES co-expression. Surprisingly, despite the analogical DTT oxidation rate as in K12 strain bioreactor, even a single DTT pulse resulted in improved RI activity per cell (paper III, figure 4). It was assumed, that besides facilitating or the folding GroEL/ES chaperone complex is also sheltering RI from the oxidative form of DTT. Indeed, the repeated

DTT pulsing had a much better effect for RI folding in GroEL/ES T7 system too (paper III, figure 4).

Interestingly, both construct groups maintained the productivity, even when DTT concentrations in the cultivation medium were exceeding 3.5 g L^{-1}. According Missiakas, this amount of DTT should be lethal for the *E. coli* cells (Missiakas et al. 1993). Contrary to K12 strain constructs, growth of which was inhibited after medium supplementation with DTT, the T7 RNA polymerase constructs with GroEL/ES co-expression continued to growth at μ of 0.1-0.15 h^{-1} even during repeated DTT pulsing. This could mean that the elevated levels of GroEL/ES chaperons act as cellular protein saviours from emerged redox disbalance and, thus, enhance cellular robustness. Previously, was reported that, the co-expression of engineered variant of GroEL/ES system improved the growth of *E. coli* K12 DH5α at low temperatures (Lee et al. 2008).

In summary, the above discussed findings resulted in the fed-batch bioreactor production processes allowing to obtained 320 mg L^{-1} of His_6RI in K12 RV308 pCU lacHis_6RI construct and 625 mg L^{-1} in ER2566 pET21bRI pGro7 corresponding to RI volumetric activities of 26550 and 80000 KU L^{-1}, respectively.

4.2.7 The *E. coli* proteome response after RI production under reducing conditions

The results presented in the paper II showed that a single DTT pulse to the stirred-tank bioreactor did not produce desired improvement of RI cytoplasmic activity in RV308 pCU lacHis_6RI construct (paper II). As mentioned in the previous sections, it was assumed that oxidative response in the *E. coli* cells could be induced due to the accumulation of oxidized DTT (see DTT oxidation rates in paper II). In order to answer this question and to evaluate the responsive protein groups, the comparative analysis of RV308 pCU lacHis_6RI cell proteomes before and after RI production in batch shake flasks and fed-batch stirred tank bioreactor were performed.

The analysis showed that, despite the different aeration conditions in the shake flasks and in stirred-tank bioreactor the medium supplementation with the reducing DTT provoked the drastic induction of oxidative-stress responsive proteins, especially in the stirred-tank bioreactor (paper IV, “Results and Discussion” section). Interestingly, even the low amount of oxidized DTT (see paper II for DTT oxidation patterns) in the shake flasks provoked induction of similar variety of oxidative-stress responsive proteins. Indeed the magnitude of induction of some oxidative-stress responsive proteins was detected much higher after the stirred tank-bioreactor process, for example, 3 fold higher induction of superoxide dismutase SodA (Paper IV, figure 1 and figure 2).

The induction patterns of proteins belonging to phosphate pentose and TCA cycle enzymes were detected quit similar after both processes. Interestingly, the magnitudes of induction of TCA cycle enzymes SucA, SucC were detected much higher after substrate-limited bioreactor process (paper IV). It was assumed, that drastic response of the TCA cycle enzymes during bioreactor process was resulted from higher metabolic load of the recombinant synthesis (Cheng et al. 2010) acidic stress, changes in redox conditions and temperature shifts (Maurer et al. 2005).

The very different induction patterns were detected of proteins belonging to amino acid, nucleotide synthesis and molecular turnover pathways (paper IV, figure 1 and figure 2). Interestingly, the RI production under reducing conditions had a clear impact on the induction of HisG and HisD proteins, belonging to the histidine synthesis pathway (paper IV).

Among the proteins belonging to “molecular transport” group, only the induction of outer membrane protein OmpA was detected after both processes. Interestingly, the RI synthesis in the shake flasks also provoked the induction of Crr, OmpC, OmpX and Sbp, i.e. proteins which previously were detected in response to antibiotics, pH changes (paper IV “Results and Discussion” section) and heterologous protein expression (Han et al. 2007). After both processes the very similar induction patterns of proteins related with proteolytic degradation and

folding, i.e. δ^{32} depending proteins were detected. It was considered that, synthesis induction of DnaK GrpE, GroEL, HtpG, HslVU, and trigger factor could be provoked by high accumulation level of RI, and/or cellular response to DTT (Gill et al. 1998, Han et al. 2007). The induction of ibpA/B, DnaK and ClpB could be provoked by RI cytoplasmic aggregation (Rinas et al. 2007).

Finally, the temperature downshift drastically induced cold-shock responsive proteins CspA/B, InfC/B, TypA and DeaD (paper IV). The higher magnitude of induction and higher variety of cold–shock-responsive proteins was detected after the bioreactor process. It was assumed that, more precise temperature control, longer incubation at the low temperature conditions and/or faster downshift in the stirred-tank bioreactor produced mentioned differences of cold-shock-responsive protein induction patterns (paper IV).

4.2.8 The reducing agents - versatile folding facilitators

The results obtained from the redox-depending folding of RI experiments prompted to test DTT for the folding improvement of several other model proteins, possessing a relatively high content of cysteins. Firstly, DTT allowed to facilitate soluble accumulation of artificially engineered and, thus, highly aggregating 200 kDa model protein RpoB – lysC (figure 1). Interestingly, RpoB (E. coli RNA polymerase, β subunit) and lysC (*E. coli* aspartate kinase III) were obtained completely soluble during the propagation in *E. coli* cells as two separate peptides (data not shown). The sequence analysis of RpoB – lysC fusion protein revealed that this large peptide is possessing 12 randomly distributed cystein residues in the molecule. It was suspected that oxidation of theses cystein residues during the recombinant production could stipulate the formation of non-native intramolecular disulfide bonds and thus promote the target protein aggregation (Ario de Marco et al. 2009).

The same production approach allowed to improve the folding of SssI methyltransferase, activity of which depends on the reduced cystein in protein catalytic centre (Rathert et al. 2007). Similar to RI,

the SssI production trails with redox manipulations improved the accumulation of active and soluble target protein, compared to the control production batch (figure 5 and figure 6). The results of these trails clearly showed, that *E. coli* redox environment in cytosol is not optimal for the protein with high content of reduced cysteins. The cytoplasmic environment of the *E. coli* is general reduced, but could get more oxidized when cells are in stationary growth phase and\or starved (Dunkan et al. 1998 and 1999, McDougald et al. 2002). That would possibly lead to the oxidation of SH residues in the target protein.

Unexpectedly, in this work was found that DTT can also facilitate the folding of proteins possessing disulfide bonds. The activity of 2 disulfide bonds possessing bovine DNaseI was highly facilitated in *E. coli* K12 construct by supplementing medium with 15 mM of DTT (figure 3 and figure 4). Interestingly, even weak reducing agent, like mercaptoethanol, rapidly inactivates bovine DNaseI *in vitro* (Price et al. 1969). It was assumed that during rapid DNaseI folding in the cytoplasmic space of *E. coli*, when the molecule dynamically undergoes various spherical changes, at the same time it is sheltered by cellular chaperons. Therefore, DTT is acting more as a disulfide bond isomeration agent, rather than an agent for disulfide bond disruption. This consideration encouraged the analogical production trails with the fusion protein of highly aggregating bovine enterokinase (EK) catalytic subunit with thioredoxin - (Trx-EK). According to previous reports the EK is requiring oxidative conditions to facilitate the proper disulfide bond formation (Collins-Racie et al. 1995, Huang et al. 2007). As it was expected, the medium supplementation with DTT improved the soluble accumulation of 11 cysteins possessing Trx-EK (figure 2).

To our knowledge, currently there are no reports describing efficient redox- dependent production processes of recombinant protein into cytoplasm or periplasm in shake flasks and/or in stirred-tank bioreactors. Indeed, it was demonstrated that externally added DTT can affect the redox state in cytoplasmic compartment of *E. coli*, as it was reported by Gill et al. in 1998. Gill and co-authors showed that the

folding and activity of chloramphenicol acetyltransferase (CAT) in the cytoplasmic space was altered due to the presence of DTT in the cultivation medium. In addition, the same group demonstrated that DTT induces the expression of chaperons and proteases (Gill et al. 1998). DTT was also utilized to activate the recombinant protein possessing non-native disulfide bonds in vivo, in *E. coli* cells (Espesset et al. 1996).

A good example showing the application possibilities of weak reducing agents in facilitating the isomeration of the disulfide bond *in vivo* and *in vitro* was reported by Raines Ronald (see patent US5910435). The method is based on the usage of organic dithiol molecules N,N'-bis(2-mercaptoacetyl)-1,2 diaminocyclohexane with specifically defined chemical properties: pKa more than 8.0 and a standard reduction potential greater than 0.25 volts. These organic dithiol molecules were used for RNase A disulfide bond isomeration in living PDI deficient yeast cells.

Finally, worth mentioning, that the folding of recombinant proteins could be facilitated by various other denaturating and osmotic stress stipulating medium additives (Fahnert et al. 2004, de Marco et al. 2005).

4.3 Conclusions and future perspectives

In this work the cultivation and synthesis important factors for the efficient RI propagation in *E. coli* soluble protein fraction were revealed. In addition, the two novel and original approaches (i) for fast scale-up of the recombinant process and (ii) for the propagation of sensitive-to-oxidation and aggregate-prone recombinant proteins were presented and demonstrated.

- In the first part of the work the fed-batch conditions were implemented already from the first phase of screening for the optimal construct, in order to shorten the development process and ensure that the selected expression constructs are relevant for the final scale-up process. The advantages of a novel fed-batch scale-up approach were clear after the failure to select the right clone or production

conditions of the same protein at the batch growth mode. The currently presented scale-up approach based on the EnBase® cultivation technique could become a routine procedure for any fed-batch process development, allowing not just to ascertain systematically the most suitable construct, but also to make fed-batch process development less laborious, time consuming and more cost-efficient.

- In the second part of the work it was found that the key factor for the efficient folding of RI in the cytoplasm and periplasmis the redox situation in these cellular compartments, which could be controlled by medium supplementation with DTT. Indeed, the strongest DTT effect on the RI folding was only when the target protein propagation was highly diminished by low synthesis temperature in the constructs with weak promoter and RBS. The medium supplementation with DTT approach proved to be quite versatile. Besides the RI, the soluble accumulation and the activity were significantly improved for several other proteins in different *E. coli* expression constructs.
- The introduction of an original oxygen limited fed-batch synthesis approach allowed to create favourable conditions for the RI folding at the high cell densities in stirred-tank bioreactor. The analogical bioreactor production protocol could be beneficial also for the cytoplasmic disulfide bond formation when the cultivation medium is highly aerated. In addition, the oxido-(re)shuffling could be further enhanced if DTT mediated redox manipulations are combined with the specific genetic modifications in the expression host, for example the co-expression of the disulfide oxi-reductease in specially engineered strains. Here, it was demonstrated that medium supplementation with DTT improved the activity of even a disulphide bond possessing protein, DNaseI, in a non-redox-modified K12 strain.
- The systematic RI production trials with the main *E. coli* chaperon systems showed that RI is a slowly folding protein, which requires folding assistance in the late folding stages. In addition, in the last part of the work the production strategy favourable for RI folding was established by combining low production temperature with the co-expression of GroEL/ES chaperon systems and generation of reduced

cytoplasmic conditions. This unique production approach served for the efficient RI folding at the high cell densities under micro-aerobic conditions, thus even 625 mg of fully active RI per litter of cultivation medium was obtained. To this date this is a highest volumetric RI yield obtained with *E. coli* constructs. The presented approaches for the recombinant production are suitable to fold and propagate the recombinant proteins requiring sophisticated folding and sensitive to redox conditions.

- The results from comparative proteome analysis shows that factors essential for RI folding, i.e. reducing and low temperature conditions drastically effects the cellular proteome of *E. coli* K12 strain. The RI production in shake flasks and substrate-limited stirred-tank bioreactor resulted in induction of proteins belonging to to carbon metabolism, oxidative stress response and heat shock/cold-shock responsive proteins. Once again the *E. coli* justified its robustness as a recombinant expression host, capable to cope with the co-expression of several recombinant proteins at very harsh conditions, and, at the same time, capable to maintain a sufficient productivity at the high cell densities under substrate-limited growth mode.
- Finally, we do believe that the *E. coli* still has a huge potential and wide application possibilities in recombinant protein production, especially when the metabolic/genetic engineering approaches allow to successfully implement the functional eukaryotic posttranslational modification and/folding pathways into the *E. coli* hosts.

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PAPER I

MICROBIAL CELL FACTORIES

RESEARCH Open Access

Novel approach of high cell density recombinant bioprocess development: Optimisation and scale-up from microlitre to pilot scales while maintaining the fed-batch cultivation mode of *E. coli* cultures

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Abstract

Background: Bioprocess development of recombinant proteins is time consuming and laborious as many factors influence the accumulation of the product in the soluble and active form. Currently, in most cases the developmental line is characterised by a screening stage which is performed under batch conditions followed by the development of the fed-batch process. Performing the screening already under fed-batch conditions would limit the amount of work and guarantee that the selected favoured conditions also work in the production scale.

Results: Here, for the first time, high throughput multifactorial screening of a cloning library is combined with the fed-batch technique in 96-well plates, and a strategy is directly derived for scaling to bioreactor scale. At the example of a difficult to express protein, an RNase inhibitor, it is demonstrated that screening of various vector constructs and growth conditions can be performed in a coherent line by (i) applying a vector library with promoters and ribosome binding sites of different strength and various fusion partners together with (ii) an early stage use of the fed-batch technology. It is shown that the EnBase® technology provides an easy solution for controlled cultivation conditions in the microwell scale. Additionally the high cell densities obtained provide material for various analyses from the small culture volumes. Crucial factors for a high yield of the target protein in the actual case were (i) the fusion partner, (ii) the use of of a mineral salt medium together with the fed-batch technique, and (iii) the preinduction growth rate. Finally, it is shown that the favorable conditions selected in the microwell plate and shake flask scales also work in the bioreactor.

Conclusions: Cultivation media and culture conditions have a major impact on the success of a screening procedure. Therefore the application of controlled cultivation conditions is pivotal. The consequent use of fed-batch conditons from the first screening phase not only shortens the developmental line by guarantying that the selected conditions are relevant for the scale up, but in our case also standard batch cultures failed to select the right clone or conditions at all.

Background

Recombinant production of proteins in heterologous hosts is today one of the key technologies to obtain protein for structural studies, functional characterisation and industrial production. The growing demand for a new therapeutic and commercial recombinant proteins provoked a rapid development of a large number of commercially available prokaryotic and eukaryotic expression systems and hosts.

Generally it is the aim to get the target protein within a short time in sufficient amounts and quality, i.e. in the native folded state. Although successful for a large number of proteins, often the overexpression of recombinant genes in the common host *Escherichia coli* results in aggregation [1], or even no product is accumulated due to

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degradation. Despite the availability of a large number of host strains, cloning vectors, fusion and coexpression partners, the optimisation procedure may be long-lasting and highly dependent on the experiences of the performing scientist or team.

Recommendations for "first to try" strategies based on the experience from tens of thousands of proteins [2] may be a good guide, however, unfortunately, such strategies often fail for secreted or more complex proteins. In this case often step by step optimisation is performed, starting either with shake flasks or with multiwell plates. The latter ones provide the possibility to evaluate in parallel a large number of conditions (see e.g. [3]). Recent developments provide even the option for on line monitoring of cultivation parameters such as pH, oxygen, or cell density [4-8].

Product yield and degree of aggregation depend on a number of factors which are highly interconnected. Strength of transcriptional induction and ribosome binding efficiency are strongly related to environmental parameters, such as the actual medium composition, the external pH and the growth temperature which also effect the specific growth rate and, overflow metabolism, i.e. acetate production. Environmental conditions rapidly change in shaken batch systems due to the exponential biomass increase and the inability control these factors, which provides variation between cultures; a problem which has been extensively discussed [2].

Furthermore, results from multiwell plate of shake flask optimisation studies are often not directly transferable to bioreactor scale cultures and industrial production, where the aim is to boost the product yield per liter [9]. The standard technology in a bioreactor to increase the cell density and thus the volumetric product yield is the fed-batch technology which is based on a strict control of the environmental conditions and much slower growth rates compared to batch cultures. To avoid repeated optimisation in different cultivation formats, the fed-batch technology should be applied already in the first multiwell scale cultivation platforms, which is not a trivial task. Recently, new innovations paved the way by applying substrate autodelivery solutions [10,11] or by the use of robot based feeding [12].

Here, for the first time, high throughput multifactorial screening of a cloning library is combined with the fed-batch type of cultivation in 96-well plates, and a strategy is proposed for direct scaling to the bioreactor scale. As an example we choose a "difficult to express" eukaryotic protein, an RNase inhibitor (RI) (50 kDa), which strongly aggregates if expressed in *E. coli*. The systematic multifactorial evaluation/screening approach of the gene-dependent genetic factors for protein expression in micro-scale was performed with a library with 45 vectors for cytoplasmic expression, which was constructed by further extending the periplasmic expression vector library from Kraft et al. [7]. The new cytoplasmic expression vector library includes five well known fusion partners and the screening strategy makes use of a luciferase based protein folding reporter system [8]. High throughput, parallel screening in 96 microwell plates for optimal expression followed by evaluation of cultivation factors in shake-flask scale was performed by using the EnBase® fed-batch cultivation technique, and the optimal process was reproduced in 10 L stirred bioreactor cultivations. The experimental results demonstrate a clear advantage for applying the fed-batch technology already during initial screening. Also they prove the straight forward transferability of microwell plate results to the bioreactor scale.

Results

Design of the screening vector library

The cytoplasmic expression vector library based on the expression vector pAK100 [13] and the destination vector pDest15 (Invitrogen, Karlsruhe, Germany) allows a site specific recombination of target genes *via* the λ phage attachment sites *att*RI and *att*RII by Gateway cloning. For the preparation of the vector library we used the plasmid vector pAK100 and two further derivates, which contain different variants (Lac_Cp, Lac_Cup, Lac_CTUp) of the native *lac*-promoter providing different levels of transcriptional strength (see figure 1). To reduce the background activity before induction all promoter variants contain in the upstream region the strong transcriptional terminator tHP and additionally a mutation in the CAP-site [13]. The pAK100 derivates where combined *via* PCR with three different ribosome-binding sites (RBS): RBS_*lac* (Genbank accession no. J01636), RBS_T7g10 (Genbank accession no. NC_001604), and a synthetic ribosome binding site (RBS_Var3, [14]). The construction of these parts in the plasmid set was earlier described in detail as a basis for the constructon of a set of plasmids for periplasmic expression [7].

In difference to the earlier set of plasmids, our constructs contained different cytoplasmic fusion partners upstream to the target gene (fused to the N-terminus of the protein), either a simple 6 × His tag, or a 6 × His tag fused with one of the following fusion partners: 6 × His-SUMO (small ubiquitin-related modifier) [15], 6 × His-Trx (thioredoxin), 6 × His-MBP (maltose binding protein MalE), 6 × His-GST (glutathion-S-transferase). Figure 1 shows the schematic summary of the combination of the functional units of the vector set with 45 different plasmids.

Screening in 96 microwell plates

At the screening stage the whole expression library containing all 45 vectors was cultivated in a 96 microwell plate by the EnBase® technology in 150 μL of MSM. Ini-

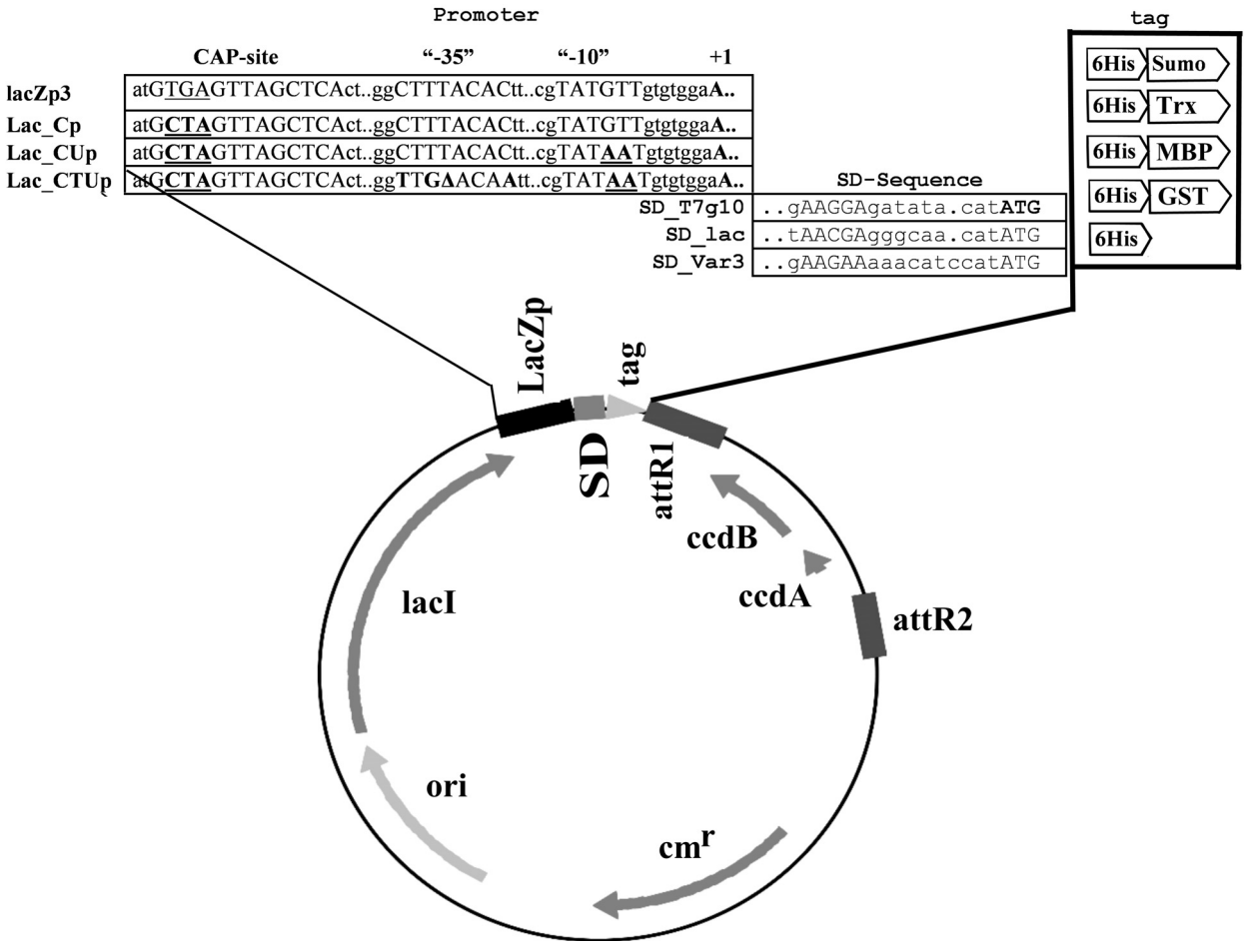

Figure 1 Schematic presentation of main expression vector library elements. The library is composed of a plasmid set of 45 vectors based on the pDEST15 vector (Invitrogen) which all contain the ColE1 origin of replication and the chloramphenicol resistance gene (*cm*^{r}) and the lacI repressor gene. The different plasmids have different *lacZp3*-derived promoters, which were generated by introducing single nucleotide mutations in the "-35" and "-10" regions (bold and underlined letters), and different ribosome binding sites. The promoter and ribosome binding site nomenclatures have been described in detail earlier [6]. In addition the expression vector library contains following target protein N-terminus tag combinations: 6 × His tag, 6 × His-GST (glutathione S-transferase), 6 × His-MBP (maltose-binding protein), 6 × His-SUMO (small ubiquitin-related modifier) and 6 × His-Trx (thioredoxin). The plasmid set is a full factorial combination of the different expression factors.

tially the cultures were started directly in Mineral Salt Medium (MSM) from the glycerol stocks. These cultures showed very high variation in cell densities (data not shown), a subject which was recently extensively discussed by Huber et al. [16].

The problem of variation was solved in our case by equalising the cultures by introducing a first "culture activation stage" directly in the gel-containing EnBase® microwell plates for 12 hours. Therefore in the first step no enzyme was added, but the culture was performed in a typical batch mode with 2.5 g L^{-1} of glucose (figure 2A). The OD_{600} values of the cultures reached 4.5 ± 1 within 12 hours. At this "synchronization point" glucoamylase (6 AGU L^{-1}) was added and thus the continuous release of glucose from the starch substrate was initiated. The enzyme controlled growth-limiting release of glucose maintained synchronized growth of the cultures to similar cell densities for further 12 hours. As a result of this simple synchronization procedure, highly similar growth patterns of all starter cultures were obtained (figure 2A) with an OD_{600} of 31 after 24 hours. This cell material was used as an inocculum for the second expression culture to which the inducer IPTG was added at an OD_{600} of 12 ± 1.0 (figure 2B). At this time all cultures had approximately the same growth rate representing a highly similar physiological state, which was important for evaluation of the parallel screening results for selection of the optimal protein expression construct.

In this first screening approach the amount of tagged RI fusions produced at either 37, 30, or 22°C in all recombinant clones was monitored at seven hours after addition of the inducer (figures 2B and 3).

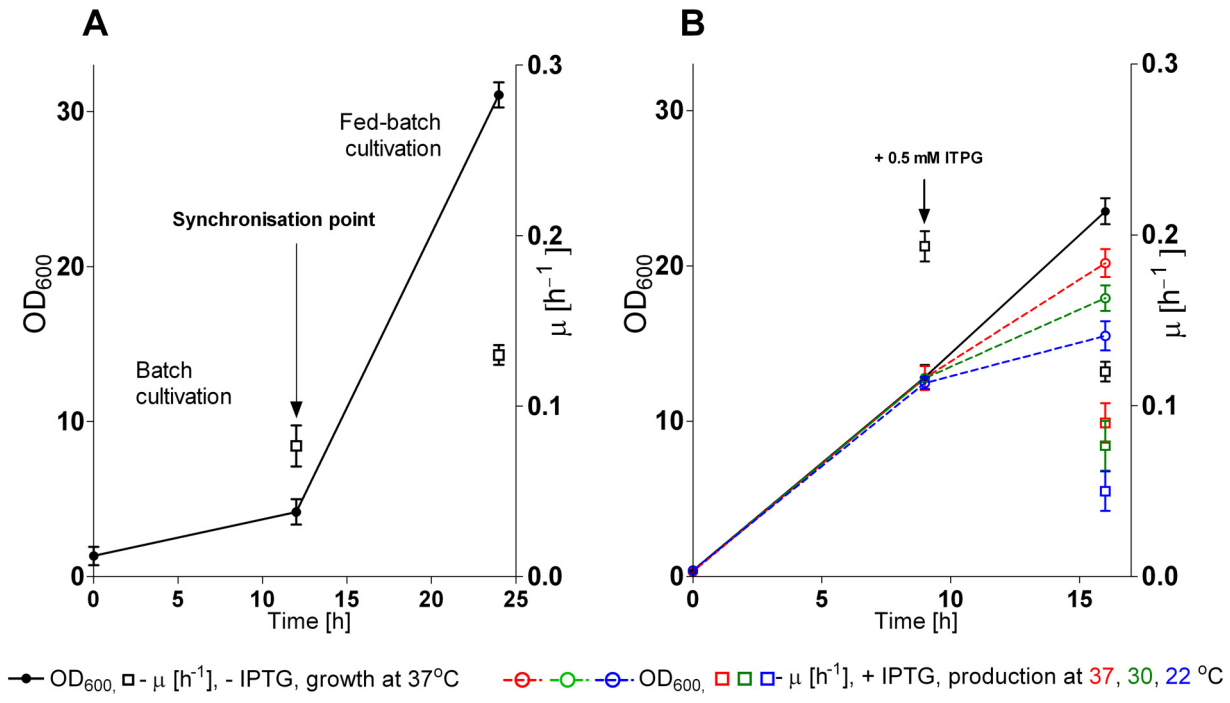

Figure 2 Growth of the RI expression library consisting of 45 different expression vectors propagated in *E. coli* RV309 p*ibpfxsT7lucA* in 96 microwell plates by the EnBase® technology. (A) Inocculum preparation and (B) 96 microwell plate expression cultures with plates which were non-induced (closed circle) or set to different postinduction temperatures at the time of induction (37°C - red, 30°C - green, 22°C - blue). Bars indicate the SD of all cultures of a whole plate (including all different vectors). OD_{600} - circles, specific growth rate - squares (average specific growth rate in the time period before).

For the evaluation of cytoplasmic accumulation of soluble tagged RI, a luminescence based robust recombinant protein folding stress reporter assay was used which was described earlier [8]. Therefore all strains contained, aside from the specific expression plasmid, also the plasmid p*ibpfxs*T7*lucA*, containing the luciferase gene under control of an *E. coli* σ^{32}-dependent tandem promoter system derived from the promoters of the *ibpAB* and *fxsA* genes [8]. As shown in the earlier paper, luciferase is strongly expressed when aggregation, i.e. inclusion body formation, occurs in the cell. In our case the measured luminescence signals were highest in the expression strain groups with the expression vectors in which the RI was fused with the tags of 6 × His, 6 × His-GST, 6 × His-Sumo, or 6 × His-Trx (figure 3A for 37°C, Additional file 1 for 30 and 22°C), implying that the product was aggregated in the cultures of these constructs. The relative luminescence between the different strains was similar in the cultures performed at 30 and 22°C compared to the cultures performed at 37°C. However, the lower luminescence values in the 22°C cultures were not due to a lower level of aggregation, but due to the lower level of expression (figure 3D and Additional file 1 part B). Interestingly, no obvious dependency of the aggregation signal at different expression temperatures on the vector promoters and/or strengths of the ribosomal binding site could be observed. Only the group of expression clones in which RI was tagged with the 6 × His-MBP fusion showed a significant lower luciferase activity independently from the construct or the production temperature, i.e. it showed lower aggregation compared to the others (figure 3, Additional file 1). The strains containing the vectors for producing 6 × His-MBP-RI fusion protein had in average a two- to threefold lower luminescence signal compared to the highest signals which was measured from the constructs which only contained the 6 × His-tag at all expression temperatures. These results indicate that MBP serves as a solubilising factor, but interestingly the soluble state is not influenced by the lower temperature or strength of synthesis (figure 3A, Additional file 1).

The data derived from the luciferase assay were confirmed by SDS-PAGE analysis of total, soluble, insoluble

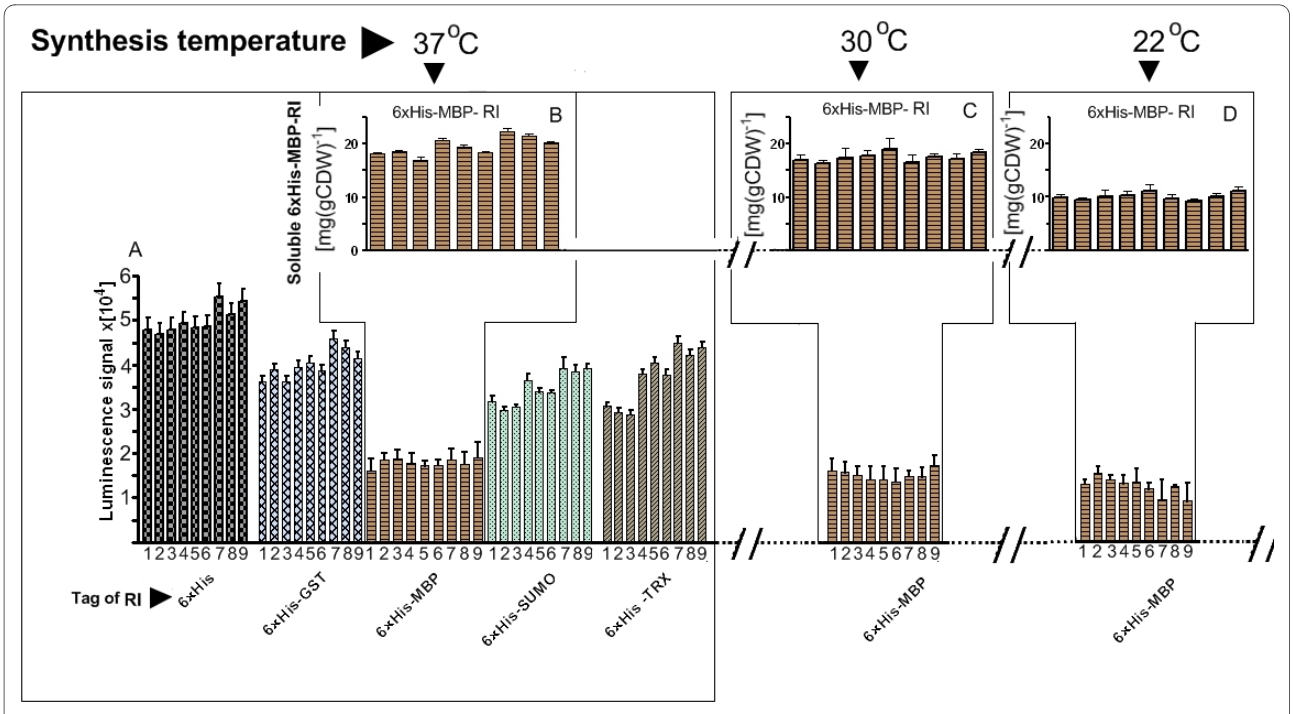

Figure 3 Aggregation signal measured as luminescence in samples of the RI expression library consisting of 45 different expression vectors propagated in *E. coli* RV309 p*ibpfxsT7lucA* in 96 microwell plates by the EnBase® technology 7 hours after induction. The lower graph (A) represents luminescence values, reflecting tagged RI protein misfolding stress levels of all expression systems 7 hours after induction with 0.5 mM IPTG. The luminescence values generated by overexpressed luciferase are derived from luciferase activity measurement from cultures performed at 37°C (full set of values displayed) fragments of results derived from cultures performed at 30 and 22°C, for which only the soluble fusion protein giving expression platforms are presented. The bars are numbered in respect to the expression system: 1 - pCT7, 2 - pClac, 3 - pCVar, 4 - pCUT7, 5 - pCUlac, 6 - pCUvar, 7-pCTUT7, 8-pCTUlac, 9-CTUVar. The upper graphs (B, C and D represent soluble fusion protein amounts in mg per gram of cell dry weight [mg $(gCDW)^{-1}$], of the cultures with the 6 × His-MBP-RI fusion constructs at 37°C 30°C and 22°C which gave the lowest luminescence signal.

protein fractions and revealed that the tagged RI was accumulated only in the soluble fraction of the clones with the 6 × His-MBP tag (Fig. 4, SDS-PAGE gel images - C1, C2 and C3). Agilent "LabChip" analysis, used for quantification of 6 × His-MBP-RI in soluble fractions, showed highly similar amounts of soluble target protein for all 6 × His-MBP-RI constructs, independently from the strength of the respective promoter or ribosomal binding site (figure 3B). As there was no obvious disadvantage of using a strong ribosome binding site, and as the strength of induction at the level of transcription later could be easily optimized by varying the amount of inducer, the expression clone containing the strongest promoter (pCTU), the T7 ribosomal binding site, and the 6 × His-MBP fusion partner was selected for further optimisation in larger cultivation scales.

Conditional screening in shake flasks

After selection of the expression construct which provided a good production of soluble 6 × His-MBP-RI protein in the microwell plate scale, the next question was whether the growth rate at the time of induction would have a major influence on the soluble expression of the target protein.

This influence of the specific growth rate was investigated by EnBase® cultivation in 1 L shake flasks with 200 mL of cultivation volume at 37°C. In order to collect reliable data for evaluation of culture behavior during the fed-batch mode in the shake flasks, several fed-batch cultivations with the EnBase® system-generated constant feeding mode were performed under strictly the same cultivations conditions. Due to the high reproducibility of shake flask fed-batch processes, it was possible to reproduce the steadily decreasing specific growth rate from the several, independent cultivation experiments (figure 5). On the basis of derived cultivation data, protein induction was performed at different time points, which each represent a different specific growth rate: $\mu_{1(t1 = 4\ h)} \approx 0.33\ h^{-1}$, $\mu_{2(t2 = 7\ h)} \approx 0.22\ h^{-1}$, and $\mu_{3(t3 = 13\ h)} \approx 0.1\ h^{-1}$ (Fig. 5A and 5B). In parallel, as a control, the same expression strain was cultured and induced under batch cultivation conditions in (i) standard LB medium, in 10 g L^{-1} of glucose containing (ii) semi synthetic and in (iii) MSM medium. In the batch cultures the target protein production was induced at OD_{600} of 1.0 at the maximum specific growth rates. The recombinant production in the cultures cultivated in LB and semi synthetic medium was

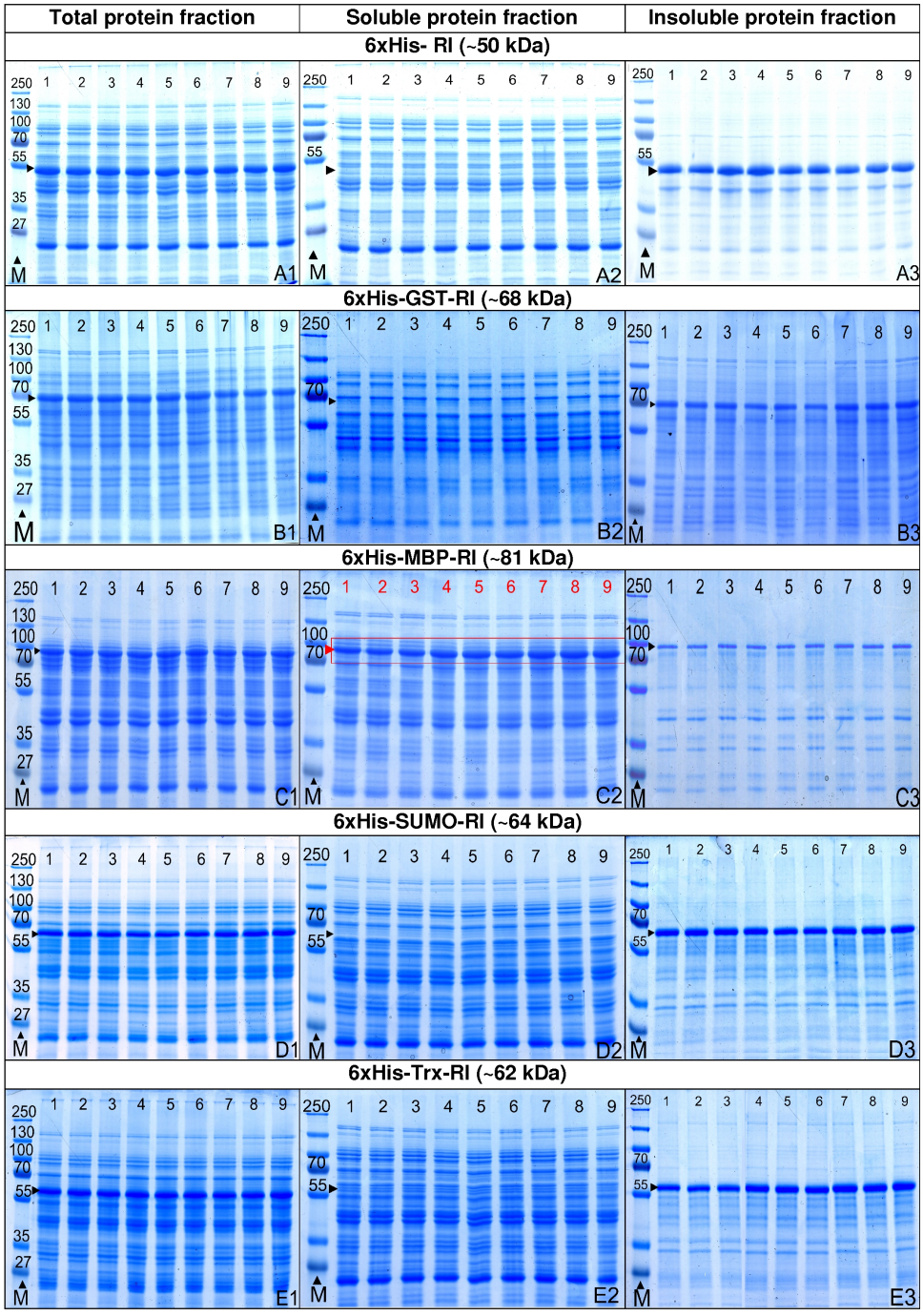

Figure 4 SDS - PAGE images of total, soluble and insoluble cellular protein fractions normalized to biomass quantity of the 7 h samples of 96 microwell plate cultures of the RI expression library consisting of 45 different expression vectors propagated in *E. coli* RV309 p*ibpfxsT7lucA* by the EnBase® technology performed at 37°C. The gel lanes are numbered in respect to the expression system: 1 - pCT7, 2 - pClac, 3 - pCVar, 4 - pCUT7, 5 - pCUlac, 6 - pCUvar, 7-pCTUT7, 8-pCTUlac, 9-pCTUVar. Rows: RI produced in total soluble and insoluble protein fractions, tagged with the 6 × His tag (size of fusion protein 50 kDa) (A1-A3); 6 × His-GST (68 kDa) (B1-B3); 6 × His-MBP (81 kDa) (C1-C3); 6 × His-SUMO (64 kDa) (D1-D3); 6 × His-Trx (62 kDa) (E1-E3). Lanes marked with letter M show the protein size marker PageRuler™ Protein Ladder Plus from Fermentas Ltd.

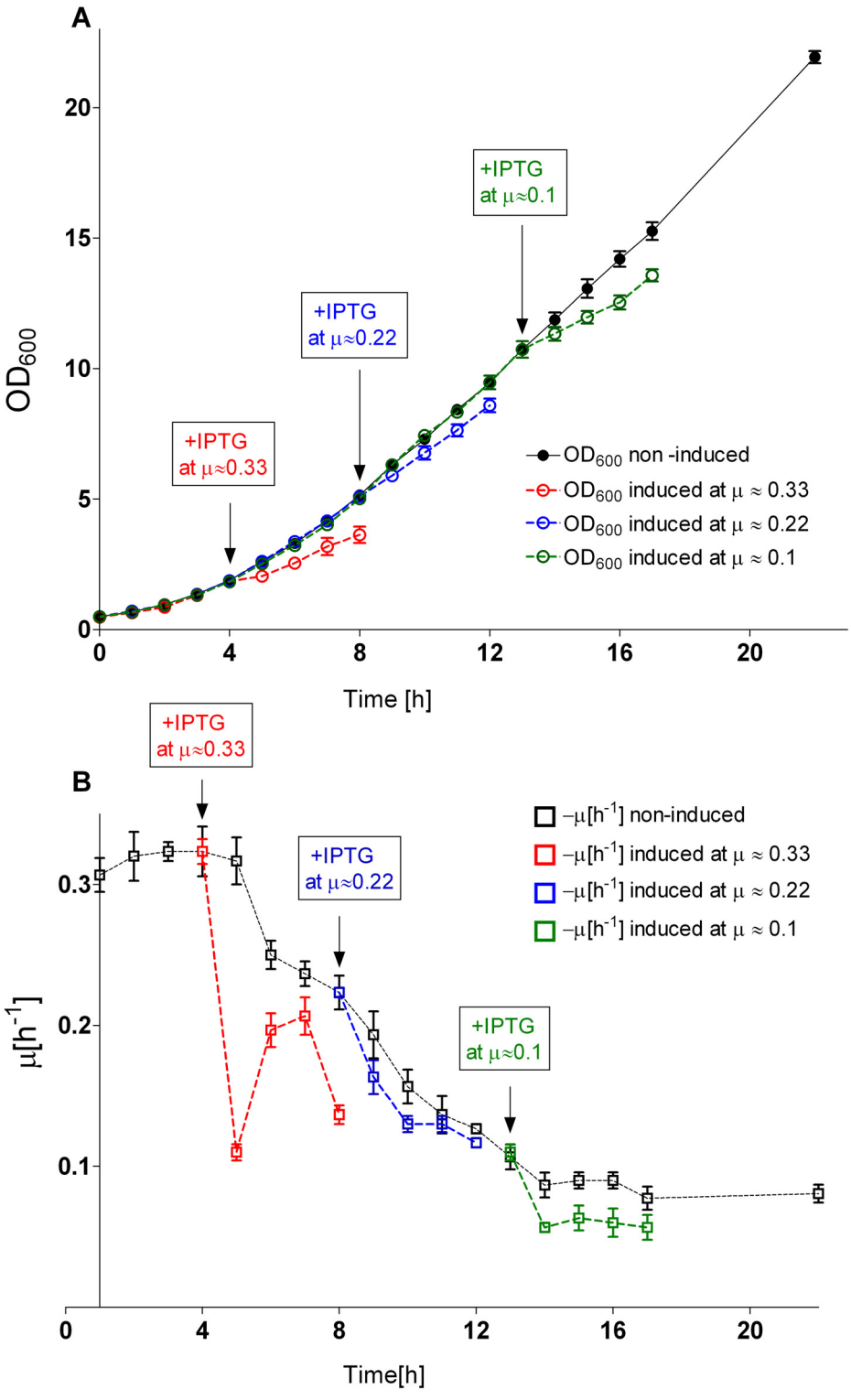

Figure 5 Shake flask fed-batch culture growth of *E. coli* RV308/p*ibpfxsT7lucA*/pCTUT7MBP-RI with and without target protein production. (A) Growth curves (OD_{600}) of independent fed-batch cultivations at 37°C and the calculated corresponding specific growth rate μ (B). Induction of 6xHis-MBP-RI synthesis was performed at the selected time points when the specific growth rate was about 0.33 h^{-1}, 0.22 h^{-1}, or 0.1 h^{-1}, respectively. The culture cultivation curves were generated from three independent cultivation experiments.

induced at μ of 0.7 h^{-1}, and in glucose-MSM medium at μ of 0.38 h^{-1} respectively. The target protein synthesis was followed for 4 hours at 37°C in analogy to the EnBase® cultures.

The results indicate a clear impact of the specific growth rate and the cultivation medium or technique on the overall yield of the 6 × His-MBP-RI product (figures 6 and 7). Interestingly, the ratio between soluble and insoluble product was rather constant under all cultivation conditions. The highest amount of soluble 6 × His-MBP-RI protein per cell unit was found in the EnBase® culture induced at a specific growth rate of 0.22 h^{-1}, which was slightly higher compared to to induction at a μ of 0.33 h^{-1} (15% lower yield) and compared to the batch culture on MSM (32% lower), (see figures 6 and 7). A significantly lower product yield (55%) was detected when the culture was induced at μ of 0.1 h^{-1}. Interestingly the yield of soluble product was lowest in the cultures which contained complex additives, i.e. in LB and semi-synthetic medium (see figures 6 and 7).

Besides evaluation of 6 × His-MBP-RI soluble protein amounts by using the LabChip approach, also RnaseA inhibitor activities were determined in the normalised crude extracts of biomasses (method according to [17]) produced in previously described fed-batch and batch synthesis experiments (figure 6, chart B). The detected ribonuclease inhibitor activities correlated with the determined soluble 6 × His-MBP-RI amounts and proved the ability of the 6 × His-MBP-RI fusion protein to inhibit Rnase A.

The results from the batch cultivation mode experiments in comparison to the EnBase® cultures indicated, unexpectedly, a lower yield of soluble 6 × His-MBP-RI protein yield per cell unit in all batch cultures (see figures 6 and 7), indicating the importance of the composition of the cultivation medium and the process control scheme. In case of our target protein it was absolutely necessary to perform the whole screening procedure with the fed-batch strategy.

Scale-up to fed-batch and batch bioreactor cultivations

Finally, as the last optimisation step, optimal 6 × His-MBP-RI protein synthesis was successfully reproduced in the 10 L bioreactor scale at different cell densities (figure 8). In order to create optimal cultivation conditions for the 6 × His-MBP-RI protein induction and production in the bioreactor, a glucose-limited fed-batch process with an exponential feeding profile to guarantee a specific growth rate μ_{set} of 0.22 h^{-1} was applied, according to the best results obtained in the EnBase® shake flask experiments. During the exponential growth phase these fed-batch cultures were induced at a OD_{600} of 9 or 31 (referred to as fed-batch process 1 and 2) and the bioreactor was harvested at 4 hours after induction at OD_{600} of 14 or 50, respectively.

The amount of the soluble 6 × His-MBP-RI protein per cell unit at the different cell densities was nearly the same in both fed-batch bioreactor runs and also the same as in the EnBase® shake flask with analog cultivation conditions (figure 9, SDS PAGE images A and B, charts D, E). The cellular productivity in the fed-batch bioreactor was maintained at the same efficiency level independently from the pre- and post induction cell densities (figure 9, images A, B and charts D, E). Thus the final volumetric product yield was higher in the fermentation where recombinant protein production was induced at the higher cell density.

The significance of specific growth and substrate uptake rates on the production of 6 × His-MBP-RI protein was also verified at the bioreactor scale by target protein production with the batch cultivation mode (figure 8C). All batch cultivation and target protein production parameters (i.e. medium composition, rate of aeration and pO_2 level, cultivation temperature) were maintained the same as in the fed-batch production processes. In batch boreactor cultures 6 × His-MBP-RI protein synthesis was induced at an OD_{600} of approximately 5 (μ = 0.45 h^{-1}) and the cultivation was continued for 4 hours at 37°C (figure 8C).

As earlier shown in the shake flask experiments, the batch production process resulted in a 40% lower production of soluble product, which was also confirmed with the activity test (figure 6). Interestingly, however the total amount of product was similar to the fed-batch process 1, but much more of the product was detected in the insoluble cell fraction. Compared to the corresponding shake flask culture, the batch bioreactor production process had a 10% higher total protein yield, but the soluble 6 × His-MBP-RI protein in bioreactor was 8% lower (figures 6, 7 and figure 9 charts D, E).

Discussion

The high throughput screening in 96 microwell plates

This study to our knowledge is the first documentation for an example of performing the development of a recombinant process from a library screening stage upwards to process fully straight by the fed-batch cultivation mode. The approach is very simple and fast. In our case all stages, including the expression library generation, could be performed in about a month's time.

Firstly, we created a new plasmid library, which is complementary to the earlier periplasmic library described by Kraft et al. [7]. The new library consists of 45 vectors which contains the three promoters of different strength, and the three ribosome binding sites from the old library, but the new plasmid set also contains 4 different N-terminal fusion partners, SUMO, thioredoxin (TRX), maltose binding protein (MBP), and glutathione S-transferase (GST). Additionally each vector contains an N-terminal 6 × His tag (see Results section, Figure 1). This library can

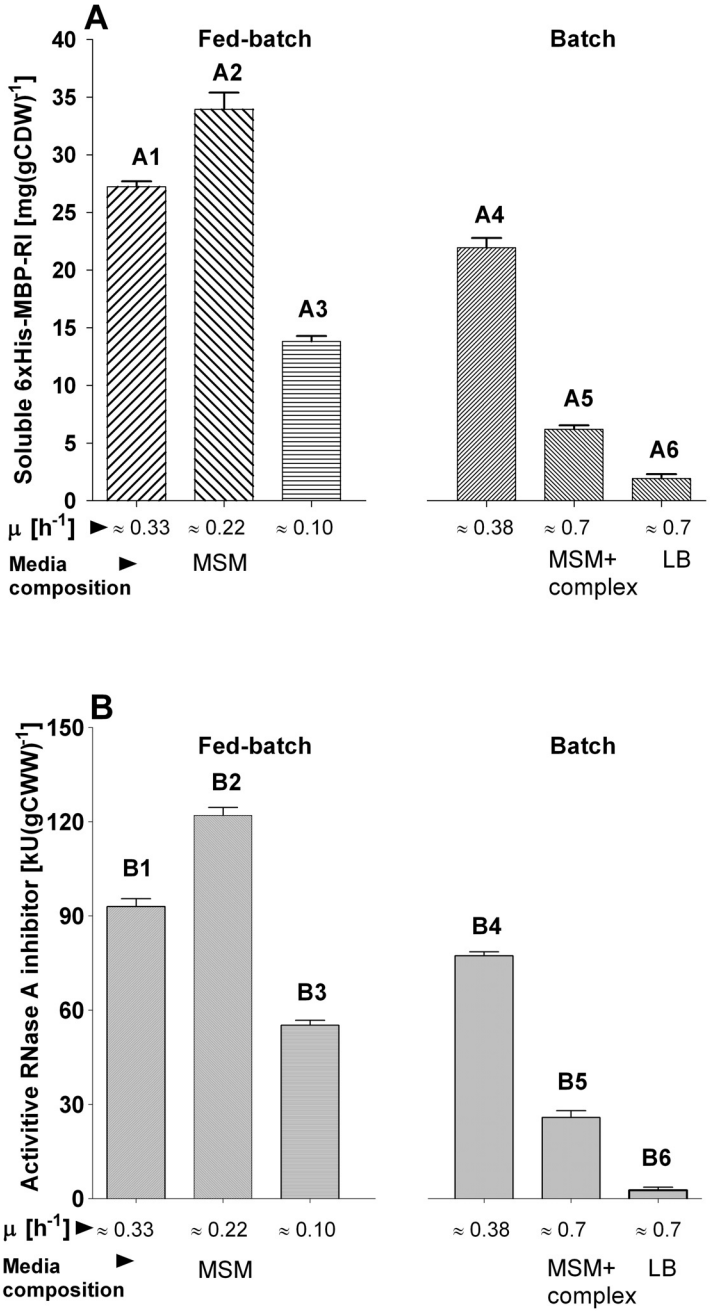

Figure 6 Chart A: amounts of soluble 6 × His-MBP-RI protein in mg per gram of cell dry weight (mg gCDW^{-1}) after shake flask batch and fed-batch cultivation with 6 × His-MBP-RI protein production at 37°C in *E. coli* RV308/p*ibpfxsT7lucA*/pCTUT7MBP-RI. Columns A1, A2 and A3 represent soluble 6 × His-MBP-RI protein amounts [mg (gCDW)$^{-1}$] obtained after 4 hours of fed-batch production at 37°C when induction was performed at different specific growth rates (bars A1-A3). Bars A4-A6 represent the amounts of soluble 6 × His-MBP-RI protein [mg (g CDW)$^{-1}$] after 4 hours of batch production at 37°C in MSM medium containing 10 g L^{-1} of glucose (column A4), semi-synthetic medium containing 10 g L^{-1} of glucose (column A5) or LB medium (column A6). Chart B: ribonuclase inhibitor activities (in kilo-units), detected in the cellular crude extracts, calculated for 1 gram of cell wet weight [kU (gCWW)$^{-1}$] after shake flask batch and fed-batch cultivation with 6 × His-MBP-RI protein production at 37°C in *E. coli* RV308/p*ibpfxsT7lucA*/pCTUT7MBP-RI. Columns B1, B2 and B3 represent ribonuclase inhibitor activities obtained after 4 hours of fed-batch production at 37°C when induction was carried at different specific growth rates (bars B1-B3). Bars B4-B4 show ribonuclase inhibitor activities after 4 hours of 6 × His-MBP-RI protein batch production at 37°C in MSM medium containing 10 g L^{-1} of glucose (column B4), semi-synthetic medium containing 10 g L^{-1} of glucose (column B5) or LB medium (column B6).

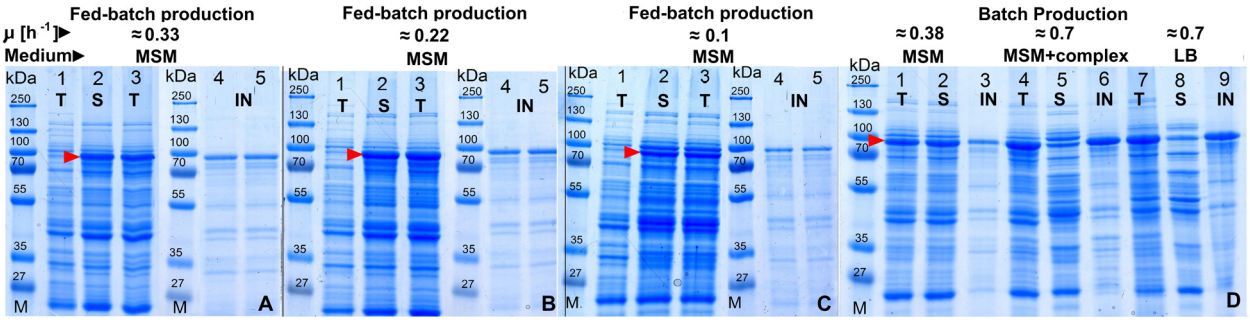

Figure 7 SDS - PAGE images of different cellular protein fractions after EnBase® fed-batch cultivation of *E. coli* RV308/p*ibpfxsT7lucA*/pCTUT7MBP-RI in shake flasks at 37°C with induction by 0.5 mM IPTG induced at different specific growth rates (A-C) and during batch production with MSM, semi synthetic medium or LB (D). Lane abbreviations: (T) - total, (S) - soluble (IN) insoluble protein fractions. Numbered SDS-PAGE gel lanes represent: 1 - total protein fraction 10 min before induction, 2 - soluble protein fraction 4 h after induction, 3 - total protein fraction 4 h after induction. Lanes 4 and 5 - insoluble protein fractions 2 and 4 h after induction. The SDS - PAGE image D represents cellular protein fractions 4 h after batch 6 × His-MBP-RI protein production when induction was carried in batch cultures of MSM medium at OD_{600} = 1. Numbered SDS-Page gel lanes represent protein fractions 4 h after induction: 1, 4 and 7 - total; 2, 5 and 8 - soluble; 3, 6 and 9 - insoluble protein fractions. The gel lanes marked with M represent the protein size marker PageRuler™ Protein Ladder Plus from Fermentas Ltd.

be combined with the luminescence based monitoring plasmid for the heat shock like response which has been described before [8].

Secondly, we used this plasmid library to screen for soluble production of a heterologous protein, an RNase inhibitor (RI), which is highly aggregating during synthesis under standard conditions (own data, not shown), which also became evident by the library screening. The screening was performed from the beginning with a fed-batch like procedure which, very surprisingly and importantly, was shown for this protein to be important. The process was successfully scaled up from 96 microwell plates to the bioreactor scale.

By utilizing the EnBase® substrate autodelivery system in 96 microwell plates, we implemented large-scale cultivation mode conditions already at the micro-scale high-throughput screening stage for the optimal RI fed-batch synthesis platform. In addition the systematic expression vector library in tandem with the monitoring system for the heat shock like response allowed to derive the key information about significant genetic features stipulating cytoplasmic accumulation of the tagged RI into the soluble fraction in very short time. The analyses of tagged RI expressed in the micro-scale cultures during the established fed-batch cultivations, showed a high robustness of this procedure towards the cultivation conditions, the cellular physiological states and the cellular synthesis capacity. Surprisingly, in contrast to our expectations, the variations in ribosome binding site and/or promoter did not significantly effect the cytoplasmic accumulation of soluble tagged RI. The only factor which yielded a higher solubility was the fusion with MBP, which was concluded to be an effective aid for RI cytoplasmic accumulation in the soluble state at the given expression conditions.

Evaluation of cultivation factors for recombinant fed-batch production

Interestingly, in difference to many other examples described in the literature, in the RI case, the amount of soluble and active product was neither influenced by the cultivation temperature nor by induction strength which was here modulated by the promoter strength. In contrast medium composition and preinduction specific growth rate had a very drastic effect on the accumulation of soluble and/or total 6 × His-MBP-RI protein.

Based on the screening data the further process development was continued with the pCTUT7 expression vector, containing the strongest promoter and ribosome binding site versions, as well as 6 × His-MBP as fusion partner.

In the shake flask scale EnBase® was applied to investigate the effect of the specific growth rate on the yield of soluble product. Therefore, simply IPTG was added at different times which reflect different specific growth rates. The highest amount of 6 × His-MBP-RI was obtained when induction was performed at a μ of 0.22 h^{-1}. Interestingly, the amount of soluble 6 × His-MBP-RI protein was lower in shake flasks performed under batch conditions with different media, which may be due to the fast growth and corresponding high product accumulation rate leading to aggregation (see Result section, figures 6, 7). In the different EnBase® cultures the lowest amount of the 6 × His-MBP-RI was obtained when cultures were induced after 14 hours of cultivation at the specific growth rate of 0.1 h^{-1} at an OD_{600} of 11. This low yield was possible due to insufficient cellular physiological activity, thus cells were incapable to maintaining high level protein production (see Result section, figures 6, 7). The results are in good agreement with optimal prein-

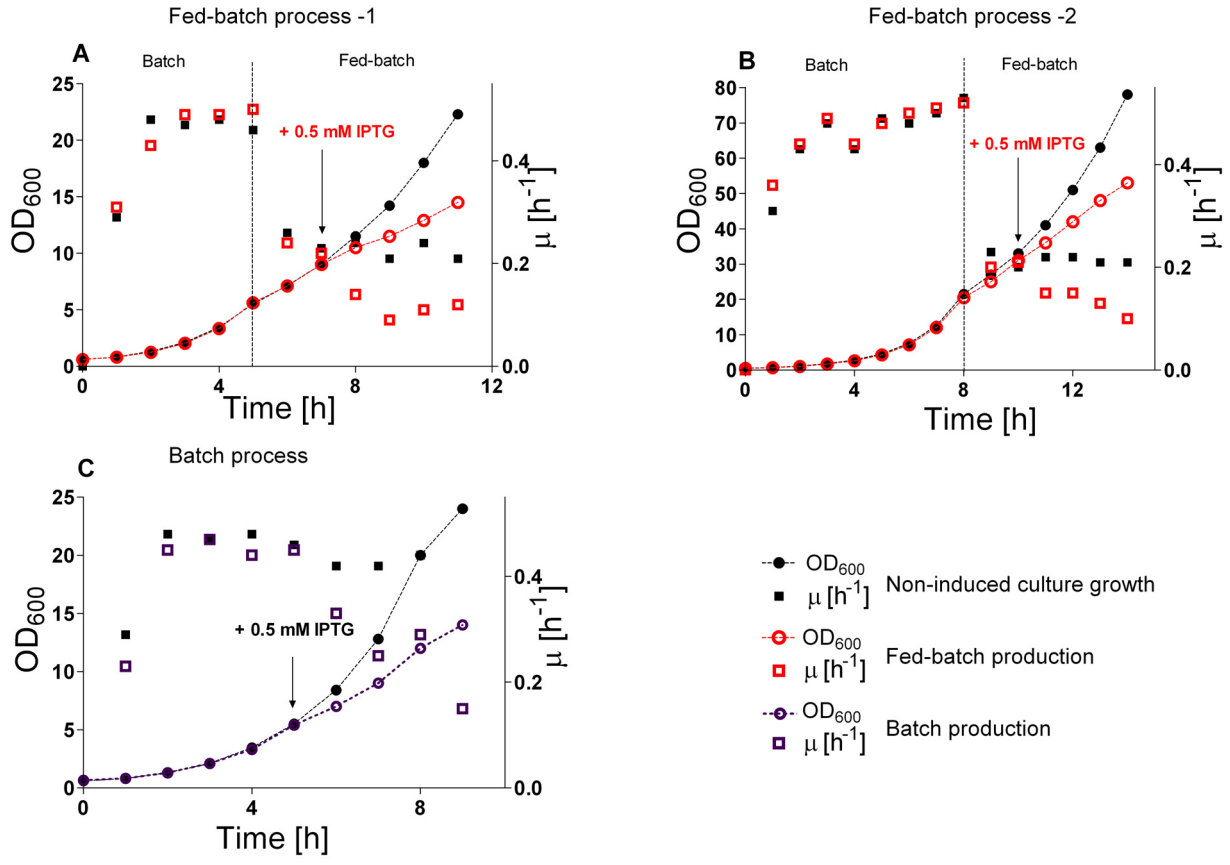

Figure 8 Growth curves of *E. coli* RV308/p*ibpfxsT7lucA*/pCTUT7MBP-RI cultures during the 6 × His-MBP-RI protein production process in a fed-batch bioreactor with exponential glucose feeding (A and B) and in a batch culture (C). Cultivations were performed at 37°C and induction was performed with 1 M IPTG. (A) Bioreactor process 1: fed-batch operation was started at OD_{600} = 6, induction at OD_{600} = 9 after the specific growth rate reached μ = 0.22 h^{-1}. (B) Bioreactor process 2; fed-batch operation was started at OD_{600} = 22, induction at OD_{600} = 31 after the specific growth rate reached μ = 0.22 h^{-1}. (C) Growth curve of the batch bioreactor process. Induction was performed at OD_{600} ≈ 5 at a specific growth rate of μ ≈ 0.45 h^{-1}.

duction growth rates detected by others, e.g. 0.2 h^{-1} by Bentley et al. [18] and 0.27 h^{-1} by Flickinger and Rouse [19] and well above the critical growth rates for growth under glucose limitation of approximately 0.15 h^{-1} [19-21] where the yield of recombinant protein production is often seen to decrease due to increased maintenance requirements and starvation responses [22-24].

Bioreactor Process

During the scale-up it was possible to maintain the cell specific production rate and, at the same time, to increase the volumetric yield by continuing the bioreactor cultivations to a higher cell density. By having information about optimal fed-batch cultivation conditions for efficient recombinant synthesis the fed-batch process was successfully reproduced even at higher cell densities in the bioreactor scale. According to the information extruded from the shake flask cultures MSM medium with a feeding mode stipulating a specific growth rate of μ = 0.22 h^{-1} was considered as optimal for the bioreactor process. The 6 × His-MBP-RI production by the fed-batch mode in shake flasks and in the bioreactor showed that by tuning the optimal substrate uptake rate we created highly similar production conditions in the EnBase® shake flasks to a stirred bioreactor. Indeed, the main difference between the fed-batch flask and bioreactor cultivation was only the higher oxygen transfer capacity in the bioreactor. Conseqently, higher cell densities could be reached, however, the specific production pattern (soluble to insoluble fraction) of the product was not influenced.

Batch cultivations in the bioreactor scale did not just allow to confirm that 6 × His-MBP-RI protein soluble accumulation is inversely related to the specific growth rate, but also indictate that the variation by scaling up from batch cultures is bigger than the variation during

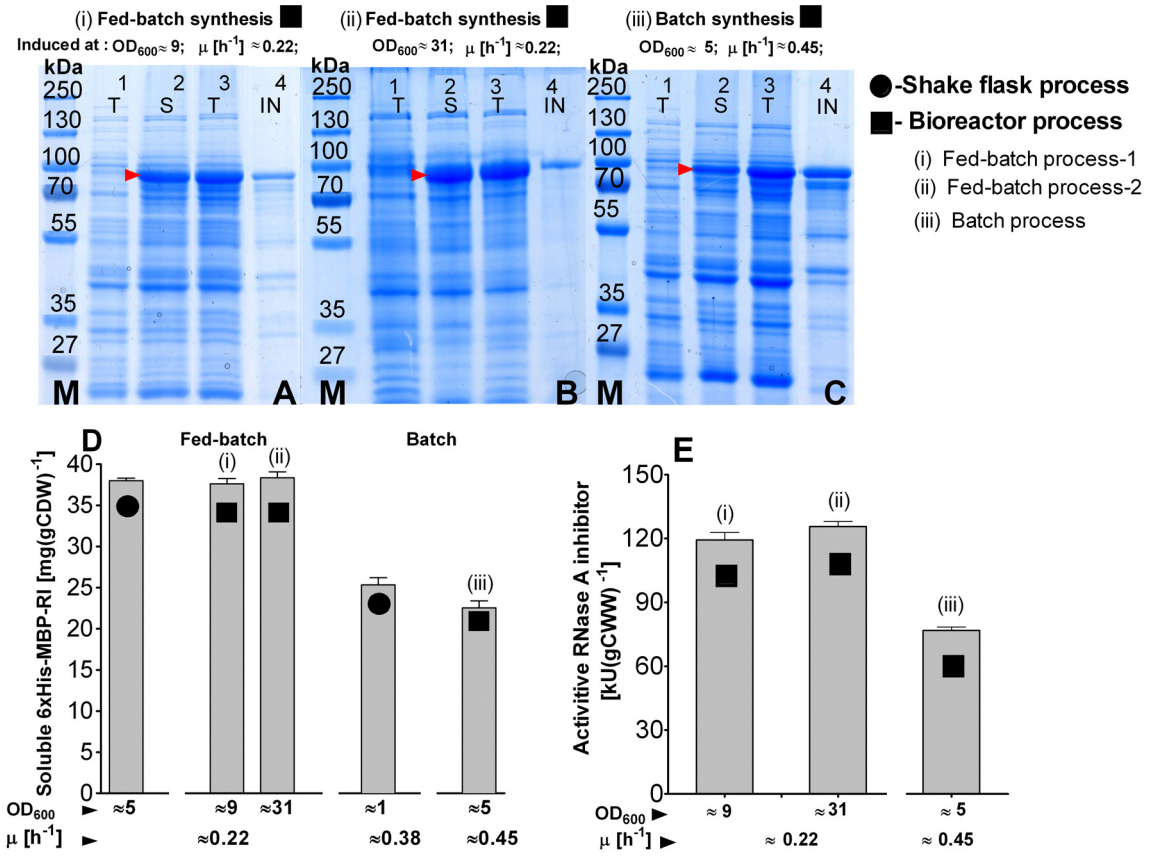

Figure 9 SDS-PAGE (A-C) and derived product amounts (D) of samples from the expression of 6 × His-MBP-RI in *E. coli* RV308/p*ibpfxsT7lucA*/pCTUT7MBP-RI fed-batch and batch cultures. (A) Bioreactor process-1, (B) Bioreactor fed-batch process-2, (C) Bioreactor batch process. Lanes: 1 - total protein fraction 10 min before induction, 2 to 4 - soluble protein fraction (2), total protein fraction (3) and insoluble protein fraction (4), all 4 hours of induction. (D) Yield of soluble 6 × His-MBP-RI in mg per gram of cell dry weight [mg (g CDW)$^{-1}$] in relation to the specific growth rate at the time of induction. Lane abbreviations: T - total, S - soluble, IN - insoluble protein fractions. Lanes marked with M: protein size marker PageRuler™ Protein Ladder Plus from Fermentas Ltd. (E) Detected ribonuclase inhibitor activities (in kilo units) in the cellular crude extracts, calculated for 1 gram of cell wet weight [kU (gCWW)$^{-1}$] after bioreactor batch and fed-batch cultivation with 6 × His-MBP-RI protein production at 37°C in *E. coli* RV308/p*ibpfxsT7lucA*/pCTUT7MBP-RI.

the scaling of a fed-batch process (see Results section, figure 9).

Our results confirm the importance of applying the fed-batch mode from the beginning of process development. Using different conditons during initial screening and later in the bioreactor scale leads to selection of conditions in the first stage which are not optimal or relevant for the second stage and would turn out in laborious optimisation steps. Currently more and more experimental appear which prove this. Clearly this conclusion is not related to *E. coli* recombinant cultures, but also has been extensively discussed in a recent paper by Scheidle et al. [25] at the example of a study with the yeast *Hansenula polymopha*. Also in this case the authors observed significant viariation of the expression of a target protein (GFP) in dependence o the cultivation conditons, and the fed-batch mode had a positive effect on the product yield.

The specific growth rate has been in many studies proven to be an important parameter for the accumulation of active and soluble product. Substrate delivery techniques like Enbase or the FeadBead system with an approximate constant rate of glucose release provide a good basis to evaluate this effect. It may be however remarked, that the volumetric yields in small scale shaken cultures are restricted by the relatively low oxygen transfer rate compared to bioreactors. However, different new approaches such as baffled microwell plates [26] or disposable mini-scale reactors with improved mixers [27,28] may also increase the yield in the small scale in future and become valuable tools to enhance the strength of screening procedures.

Conclusions

In this work we demonstrated that the high-throughput screening approach based on fed-batch type cell cultures in shaken 96 microwell plates is a suitable tool for a fast, cheap, and systematic bioprocess development based on parallel evaluation of a large number of expression platforms at uniform cultivations conditions.

During the development of the recombinant fed-batch process, by employing the EnBase® technology in 96 microwell plates and shake flasks, several important practical observations may be discussed. Firstly, as it was learned from later EnBase® microwell plate cultivations (results not shown) the first culture synchronization step is not necessary - the cultures are perfectly synchronized after 16-24 hours fed-batch cultivation due to the controlled glucose release if a small amount of glucose is added initially. Indeed it would be even more beneficial for the synchronized cultivation if the stock culture is produced from culture suspension after the uniform cultivation in liquid medium. In our case, due to the large number of expression clones we prepared the glycerol stock cultures directly from the transformation plates. Thereby it was more risky that a direct culture synchronization without a preculture would succeed. However in a new variant, EnBase®-Flo [29], it should be possible to add the biocatalyst after an initial small batch phase, which would also for libraries make the preculture step obsolete.

The amount of material for analysis of protein synthesis in 96 microwell plates is quite low. Therefore, the samples were restricted to the point of induction and to the final point of cultivation, which also resulted in less disturbance of the culture, which would have been caused by regular sampling. Despit this we could show in this study that fed-batch cultivations in the 96 microwell plate and shake flask scale are very similar. As a intermediate scale of cultivation we now use widely 24 deepwell plates, which provides the benefits of parallel screening in combination with a larger cultivation volume wich allows more analyses and due to a lower impact of evaporation a more robust process.

Our results derived from batch and fed-batch processes in different scales clearly showed for the RNase inhibitor that cultivation factors, aside from the vector construct, have a key impact on the yield of soluble product. Interestingly, controling of the specific growth rate was much more important than regulating genetic factors - promoter strength and ribosome binding sites. It remains interesting to see whether this holds true also for other proteins.

In addition and most importantly, for the RNase inhibitor traditional approaches with the screening of the library with complex medium would have totally failed to select the right clones. The use of mineral salt medium in combination with the fed-batch technology turned out to be absolutely critical for our success to develop a robust process. We believe that our results may be very relevant and transferable to many other screening studies which are currently performed with complex media as a standard procedure and therefore may miss favourable clones.

Methods

Vector library preparation

The RI encoding gene was designed for insertion into an expression vector library via site specific recombination reaction based on the Gateway® cloning technology by PCR end extension (with High Fidelity PCR mix from Fermentas) according Invitrogen's Gateway cloning manual (for details see: http://www.invitrogen.com). The PCR fragment was purified from 1% agarose with the QIAquick Gel Extraction Kit (Qiagen) and inserted into the pDONR201™ (Invitrogen) vector via Gateway® cloning "BP recombination". The whole Gateway® BP recombination reaction was transformed into the *ccdB* gene effect sensitive *E. coli* DH5α strain via calcium transformation. The halves of the transformation mixture were plated on Luria broth (LB) plates with 50 μg mL^{-1} of kanamycin. The pDONR201™ vector, containing the target gene, was purified with the GeneJET™ Plasmid Miniprep Kit (Fermentas) and used for "Gateway® LR" recombination for insertion of the target gene into to the destination protein expression library. The recombination mixture was transformed into the *ccdB* sensitive *E. coli* DH5α strain via calcium transformation and plated on LB solid medium with 30 μg mL^{-1} of chloramphenicol. The expression vectors, containing the target gene, were purified with the GeneJET™ Plasmid Miniprep Kit (Fermentas) and used for the following transformations.

Preparation of target protein expression strain library

The expression strain *E. coli* K12 RV308 (ATCC 31608) was first transformed with the reporter plasmid *pibpfxsT7lucA* previously described by Kraft et. al. [8], carrying a resistance for ampicillin, and plated on LB agar with ampicillin (100 μg mL^{-1}). The expression strain *E. coli* RV308 *pibpfxsT7lucA* was co-transformed with the RI gene containing the cytoplasmic expression library; the transformants were plated on LB agar containing ampicillin (100 μg mL^{-1}) and chloramphenicol (30 μg mL^{-1}). Both transformations were based on the calcium temperature shock method. The cell stock was produced by washing the transformants from the surface of the agar plates with 2 mL of glucose-based mineral salt medium (MSM), containing the antibiotics and glycerol (25%). The collected cell suspensions having cell densities (OD_{600}) of about 10 to 30 were aliquoted into sterile PCR stripes and stored at -70°C.

Cultivation media

Transformations and plasmid propagations were performed on solid and liquid LB medium containing Bacto-Tryptone (10 g L^{-1}), Bacto-yeast extract (5 g L^{-1}), NaCl (10 g L^{-1}), 15 g L^{-1} bacto agar (if solid medium) and the required antibiotics. Fed-batch and batch cultivations were performed in glucose-based mineral salt medium (MSM) with the following composition (per litre): Na_2SO_4 2 g, $(NH_4)_2SO_4$ 2.68 g, NH_4Cl 0.5 g, $KHPO_4$ 14.6 g, $NaH_2PO_4 \times H_2O$ 3.6 g, $(NH_4)_2$-H-citrate 1.0 g, and glucose 2.5 to 15 g. NaOH (40%) was used to adjust pH to 7.0 prior to the heat sterilisation. Semi-synthetic medium was based on the MSM with additional 10 g L^{-1} of yeast extract and 10 g L^{-1} casamino acids. Additionally, before cultivation on the mineral salt and semi-synthetic media the following sterile solutions were added: 2 mL L^{-1} of (1 M) $MgSO_4$ and 2 mL L^{-1} of trace element solution with the following composition (per litre): $CaCl_2 \times 2H_2O$ 0.5 g, $ZnSO_4 \times 7H_2O$ 0.18 g, $MnSO_4 \times H_2O$ 0.1 g, Na_2-EDTA 20.1 g, $FeCl_3 \times 6H_2O$ 16.7 g, $CuSO_4 \times 5H_2O$ 0.16 g, $CoCl_2 \times 6H_2O$ 0.18 g; as well as 100 μL L^{-1} of thiamine hydrochloride (1 M), 1 mL L^{-1} of ampicillin (100 mg mL^{-1}) and 1 mL L^{-1} of chroamphenicol (30 mg mL^{-1}). The feeding solution for fed-batch cultivations was based on fully formulated MSM with the required antibiotics and 550 g L^{-1} of glucose.

Fed-batch mode cultures and recombinant protein synthesis in 96 microwell plates

EnBase® technology (BioSilta Oy, Oulu, Finland) based fed-batch 96 microwell plate cultures were performed in 96 well flat bottom plates (Perkin Elmer). The wells of the plates were filled with heat sterilized gels, referred as - "bottom" and "top" phases [11]. Firstly, 100 μL of "bottom" gels (1.5% of Bacto-agar, Difco) and 10% of potato starch (Sigma) were added. After solidification of the bottom gels, 50 μL of top-gels (3.25% Bacto-agar) were added. The wells for cultivation were filled with 150 μL of fully formulated sterile MSM containing 100 μg mL^{-1} of ampicillin and 30 μg mL^{-1} of chloramphenicol. For cultivation the plates were closed with a plastic lid. All microscale pre-induction cultures were performed by intensive shaking with a Variomag® Thermoshake (Inheco, Germany) at 37°C and 750 rpm (amplitude 1.5 mm). The inoculum plate with the gel-based EnBase® system was inoculated with 5 μL of glycerol stock cultures per well. Synchronization of all 45 RI expression strain cultures was performed in these plates in the batch mode in MSM containing 2.5 g L^{-1} of glucose at 37°C for 12 hours (overnight). After overnight cultivation at the obtained cell densities (OD_{600} about 4.5 ± 1.0) the release of glucose from starch was started by addition of 5 μL of amylase (BioSilta Oy, Oulu, Finland) to obtain a final concentration of 6 AGU L^{-1} (amyloglucosidase units per liter). 1 AGU is the amount of enzyme which releases 1 μmol min^{-1} of maltose).

Additionally the culture suspensions were supplemented with 5 μL of NH_4OH (25%). The inoculum cultures were cultivated for another 12 hours under glucose limitation at 37°C. After the initial cultivation 5 μL of synchronized and adapted precultures were transferred to the gel-containing wells of a new microwell plate (with 6 AGU L^{-1}), from the beginning possessing linear glucose auto-release and cultivated for 9 hours at 37°C. Target gene expression was induced after 9 hours of cultivation at OD_{600} = 12 ± 0.5 by addition of 5 μL of IPTG, dissolved in fully formulated medium, to achieve a final concentration of 0.5 mM. At the time of induction also the cultivation temperature was shifted to either 37°C, 30°C, or 22°C, respectively. In all experiments the cultures were harvested 7 h after induction.

Determination of cell growth in microwell plates

The initial (0 h time point) optical density (OD) of all microwell plate cultivation samples, was determined by measuring the turbidity with a Victor³ plate reader (PerkinElmer) with the following settings: wave length 490 nm, 15-20 sec shaking before plate reading. Cell densities from EnBase® cultures were determined by 30 fold dilution of 5 μL broth samples in clear deionized water or cultivation medium in a final volume of 150 μL in clear, flat bottom 96 microwell plates (PerkinElmer). The OD_{490} obtained by using the Victor³ were recalculated to OD_{600} with a 1 cm path length using the following equation, obtained by a calibration curve:

$$OD_{600} = (4.7414 \times OD_{490} - 0.1416) \times D_f,$$

where D_f is the dilution factor.

Fed-batch mode cultivations in shake flasks

The EnBase® technology based fed-batch shake flask cultivations were performed in 1 L baffled Erlenmeyer flasks in 200 mL of MSM. These shake flasks contained 100 mL of the "Bottom gel" (10% potato starch, 5% Bacto-agar) and 75 mL of the "Top gel" (5% Bacto-agar). The inoculum for the production cultures was prepared by overnight batch cultivation at 37°C in 100 mL of MSM containing 7 g L^{-1} of glucose and the appropriate antibiotics. For inoculation the cultures were washed and resuspended in 50 mL of fully formulated sterile MSM without glucose. 10-12% from the total cultivation volume of culture suspension was transferred to MSM EnBase® cultivation flask to achieve final volume of 200 mL, with the appropriate antibiotics and no glucose. 1 mL of glucoamylase, diluted in sterile deionized water, was added to the cultivation medium, just after inoculation to obtain a final amylase concentration of 12 AGU L^{-1}. The cultiva-

tions were performed at 37°C and 180 rpm on a Multitron shaker with an orbit of 2.5 cm (Infors). Product - 6 × His-MBP-RI synthesis was induced at three different time points (t_1 to t_3) which corresponded to the following specific growth rates: t_1 = 4 h, OD_{600} = 2.0 ± 0.2, $\mu_1 \approx 0.33$ h^{-1}; t_2 = 8 h, OD_{600} = 5.5 ± 0.2, $\mu_2 \approx 0.22$ h^{-1}; and t_3 = 13 h, OD_{600} = 11 ± 0.2, $\mu_3 \approx 0.1$ h^{-1}. Induction was performed by addition of IPTG (1 M) to achieve a final concentration of 0.5 mM. The protein was produced for 4 h, at 37°C, shaking at 180 rpm.

Batch mode cultivations in the shake flasks

The inoculums for batch protein production in the shake flasks were prepared by overnight batch cultivation of the selected clone in 100 mL volume shake flask with 10 ml of LB at 37°C. For protein production, 1% of the corresponding inoculum culture was transferred either to fresh LB, or to mineral salt medium, or to a semi synthetic medium, both containing 10 g L^{-1} of glucose, to a final volume of 200 mL in 1 L baffled Erlenmeyer shake flasks. Cultures were cultivated at 37°C and 180 rpm until they reached the induction point, corresponding to a cell density of OD_{600} = 1 ± 0.05. Induction was performed by addition of IPTG (1 M) to a final concentration of 0.5 mM. The MBP-RI fusion was synthesized for 4 h, at 37°C.

Bioreactor processes

Batch and Fed-batch cultivations were performed in a 10 L working volume Biostat C bioreactor (with MFCS/win 2.0 supervisory system, B. Braun Biotech, Melsungen, Germany) with the following parameters: the pO_2 was maintained at 30% by adapting the stirrer rate and automatic regulation of the air flow (from 0 to 30 liters per min), the cultivation temperature was 37°C (before and after induction), pH was controlled at 7.0 ± 0.1 by addition of NH_4OH (25%) or H_3PO_4 (2 M). MSM containing 15 g L^{-1} of glucose was used in the batch production process.

The fed-batch cultivations were started with a volume of 8.5 L of MSM, and contained 4.5 g L^{-1} and 15 g L^{-1} of glucose, respectively. The feeding was controlled by the Biostat software (version 4.62).

Exponential feeding profiles were programmed to maintain a specific growth rate of $\mu \approx 0.22$ h^{-1}. The feeding profiles were calculated with the following equations:

$$F(t) = F_o e^{\mu t}$$

where F_o is the initial feeding rate [L h^{-1}], μ is the specific growth rate [h^{-1}] to be maintained during feed operation, and t is the time after feed start [h]. The initial feeding rate was calculated from the mass balance on substrate according to.

$$F_o = \frac{\mu X_0 V0}{S_f Y_{x/s}}.$$

Here, X_0 and V_0 are the cell dry weight (CDW) [g L^{-1}] and the culture volume [L] at the time of the feeding start, respectively, S_f[g L^{-1}] is the substrate concentration in the feeding solution, and $Y_{x/s}$ is the yield coefficient (g CDW per g of glucose). $Y_{x/s}$ in all calculations was 0.3 g g^{-1} as calculated from batch fermentations.

Before initiation of the fed-batch mode cells were cultivated as a batch until $OD_{600} \approx 6$ or ≈ 22, respectively. The biosynthesis of the product was induced during the fed-batch mode at $OD_{600} \approx 9$ or $OD_{600} \approx 31$, respectively. The specific growth rate at the time of induction was the same in both cases, 0.22 h^{-1}. After induction by 1 M IPTG cultivations were continued for 4 h at 37°C under continuation of the exponential feed function. In the batch process, performed as a control, 6 × His-MBP-RI target protein synthesis was induced at $OD_{600} \approx 5$ ($\mu \approx 0.45$ h^{-1}) and the culture was continued for 4 hours at 37°C.

Luciferase assay-target protein misfolding stress monitoring

After OD_{600} determination the analyzed bacterial cultures were separated from the cultivation medium by centrifugation in a microcentifuge (14000 rpm, 5 min). The cell pellet was resuspended in 0.9% NaCl and diluted to achieve 8 × 10^7 cells mL^{-1} (corresponding to 0.10 OD_{600}) in a final suspension volume of 200 μL. The whole 200 μL suspensions were transferred to the wells of a 96 microwell plate (Greiner) with transparent bottom. Then 100 μL of fresh reaction buffer (25 mM tricine, 15 mM $MgCl_2$, 5 mM ATP, 7 mM beta-mercaptoethanol, 0.5 mg mL^{-1} bovine serum albumin, 13 mM D-luciferin Na-salt, pH 7.8) were added and the luminescence was measured with a Victor^3 multilable counter (Wallac) every 10 min at 25°C over a total time of 60 min. Blank samples were included (expression strain cultures cells cultivated without induction). The "true" luminescence values were calculated for each sample from the average of the measured values during the plateau phase by applying the formula:

$$\mathrm{Tv} = (\mathrm{Mv} - \mathrm{Bv}) \times \mathrm{Df} \times \mathrm{N}$$

where Tv - True, Mv - Measured and Bv - Blank luminescence values calculated by formula Bv = (Blank signal) × Df × N, Df - dilution factor and N - normalization factor for the cells amount corresponding to cell density of 1 at OD_{600}.

Protein analysis

For visualization and quantification of the soluble and insoluble protein fractions from microwell plate cultures,

normalised amounts of cellular suspension were transferred to 1.5 mL Eppendorf tubes, harvested by centrifugation (10 min, 14000 rpm, 4°C) and the pellet was resuspended in 100 μL of lysis buffer (50 mM Tris-HCl pH 8.0, 0.1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 5 mM DTT, 0.1 mg mL^{-1} lysozyme). The whole cellular suspensions were sonicated for 10 sec with a Vibra cell™ sonicator (Sonic and Materials Inc., 2 mm diameter probe tip) at 4°C. The supernatant (soluble fraction) was collected after centrifugation (10 min, 14000 rpm, 4°C) and the pellet (insoluble protein fraction) was resuspended in 100 μL of lysis buffer without lysozyme. Samples for SDS-PAGE were prepared as follows: 25 μL of 5× SDS-PAGE loading buffer, 5 μL of 20 × DTT (Fermentas Ltd.) and 20 μL of deionized water were added to 50 μL of the respective protein suspensions in order to obtain a final sample volume of 100 μL. Samples were heated for 15 min at 95°C. 10 μL of sample was applied to each lane of a 10% SDS-PAGE gel.

Cell samples harvested from flask and bioreactor cultivations were resuspended in lysis buffer with the following ratio: 1 g of biomass with 5 mL of lysis buffer. Lysis was performed by sonication for 30 sec (Vibra cell™, Sonic and Materials Inc., 6 mm diameter probe tip) at 4°C. The lysate was distributed to 1 mL fractions and centrifuged (10 min, 14000 rpm, 4°C). The supernatant (soluble protein fraction) was collected and the insoluble protein fraction containing pellets from the 1 mL disrupted cell suspensions were resuspended as described above in 1 mL lysis buffer without lysozyme. 10 μL of soluble protein and cellular debris suspensions were taken for SDS-PAGE sample preparation. Final volumes of 100 μL of SDS-PAGE samples were obtained as described above and 10 μL of these suspensions were applied for the SDS-PAGE run.

Quantification of the target protein in soluble protein fractions was performed after separation on an Agilent 2100 bioanalyzer. Therefore the normalised crude extracts were 4-fold diluted in the buffer containing 50 mM of Tris-phosphate pH 8.0 and 1 mM EDTA. Evaluation of protein amounts on SDS-PAGE gels was performed by using TotalLab software (Total Lab Systems). The ribonuclease inhibitor activity in the normalised for the biomass quantity crude extract was determined by activity assay described by Blackburn et al. [17].

Additional material

Additional file 1 Supplementary figure 1. Aggregation signal measured as luminescence in samples of the RI expression library consisting of 45 different expression vectors propagated in *E. coli* RV309 p*ibpfxsT7lucA* in 96 microwell plates by the EnBase® technology 7 hours after induction for the cultures performed at 30°C (A) and 22°C (B). Bars are numbered in respect to the expression system: 1 - pCT7, 2 - pClac, 3 - pCVar, 4- pCUT7, 5 - pCUlac, 6 - pCUvar, 7-pCTUT7, 8-pCTUlac, 9- CTUVar.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
JS designed the experimental setup, performed all cultivation experiments and prepared the manuscript. JPP helped with the screening set-up and RR contributed to the bioreactor experiments and product analysis. MK and UH constructed the cytoplasmic expression library. PN initiated the project, assisted with data analysis and manuscript preparation. All authors read and approved the final manuscript.

Acknowledgements
The work was performed in relation with the UNICAT Center of Excellence at the TU Berlin. The research project was fiancially supported by a research grant of Fermentas UAB.

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Received: 23 March 2010 Accepted: 20 May 2010
Published: 20 May 2010

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doi: 10.1186/1475-2859-9-35
Cite this article as: Šiurkus *et al.*, Novel approach of high cell density recombinant bioprocess development: Optimisation and scale-up from microlitre to pilot scales while maintaining the fed-batch cultivation mode of E. coli cultures *Microbial Cell Factories* 2010, **9**:35

PAPER II

MICROBIAL CELL FACTORIES

RESEARCH Open Access

Reducing conditions are the key for efficient production of active ribonuclease inhibitor in *Escherichia coli*

Juozas Šiurkus[1] and Peter Neubauer[2*]

Abstract

Background: The eukaryotic RNase ribonuclease/angiogenin inhibitors (RI) are a protein group distinguished by a unique structure - they are composed of hydrophobic leucine-rich repeat motifs (LRR) and contain a high amount of reduced cysteine residues. The members of this group are difficult to produce in *E. coli* and other recombinant hosts due to their high aggregation tendency.

Results: In this work dithiothreitol (DTT) was successfully applied for improving the yield of correctly folded ribonuclease/angiogenin inhibitor in *E. coli* K12 periplasmic and cytoplasmic compartments. The feasibility of the *in vivo* folding concepts for cytoplasmic and periplasmic production were demonstrated at batch and fed-batch cultivation modes in shake flasks and at the bioreactor scale.
Firstly, the best secretion conditions of RI in the periplasmic space were evaluated by using a high throughput multifactorial screening approach of a vector library, directly with the Enbase fed-batch production mode in 96-well plates. Secondly, the effect of the redox environment was evaluated in isogenic *dsbA*$^+$ and *dsbA*$^-$ strains at the various cultivation conditions with reducing agents in the cultivation medium. Despite the fusion to the signal peptide, highest activities were found in the cytoplasmic fraction. Thus by removing the signal peptide the positive effect of the reducing agent DTT was clearly proven also for the cytoplasmic compartment. Finally, optimal periplasmic and cytoplasmic RI fed-batch production processes involving externally added DTT were developed in shake flasks and scaled up to the bioreactor scale.

Conclusions: DTT highly improved both, periplasmic and cytoplasmic accumulation and activity of RI at low synthesis rate, i.e. in constructs harbouring weak recombinant synthesis rate stipulating genetic elements together with cultivation at low temperature. In a stirred bioreactor environment RI folding was strongly improved by repeated pulse addition of DTT at low aeration conditions.

Background

Escherichia coli is the most widely used host for recombinant protein production. Aggregation of the target protein in *E. coli* is a common phenomenon which is a consequence of the inability of the host's folding machinery to cope with the rapidly accumulating target protein folding and/or to facilitate efficient stabilization of SH groups, or to contribute to the formation and/or reorganization of correct disulfide bonds.

Contrary to most cases reported in literature, which focussed on the enhancement of disulfide bond formation in recombinant proteins by modulating the redox situation, we found it challenging to improve the folding of eukaryotic ribonuclease inhibitor RI (~49 kDa) which is characterised by a high amount of reduced cysteins, which are vital for the function of the protein. Our model protein - RI, shows a homology of 79-82% to the well characterized RNase ribonuclease/angiogenin inhibitors from human (hRI), rat (rRI), mouse (mRI) and porcine (pRI). The members of the ribonuclease inhibitor group represent a specific subfamily within the large group of proteins with a very special protein fold - the leucine-rich repeat (LRR) proteins [1]. LRR proteins

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share very interesting features which makes them a unique group of proteins. RI has an unusual non-globular flexible horseshoe like structure, which is very conserved between different species. The core of RI molecules is composed of hydrophobic 15-16 LRR motifs. Each of the LRR's consists of a structural unit of 28 to 29 amino acids forming an α-helix and β-strand connected by loops [2]. RI has a very high leucine content (18%), but also contains 30-32 cysteine residues (6.5-7%). In difference to other LRR motif containing proteins where the cysteins are structural units (see e.g. [3]), all cysteins in RI are reduced which is very important for activity, i.e. substate interaction. Oxidation of free SH groups in RI is highly cooperative and leads to inactivation and even denaturation [2,4].

Production of RI has been a challenge due to its flexible structure, repetetive amino acids and reduced cysteins. Thus so far reported RI production attempts in the yeast *Saccharomyces cerevisiae* [5] and in *E. coli* [6,7] resulted in a low overall yield either due to a low production level and/or high RI insolubility, respectively. So far about 10 mg of active porcine RI (pRI) per liter of culture medium was produced by using the P_{trp} promoter in the *E. coli* host [7].

Recently, after high throughput multifactorial screening of an *E. coli* plasmid vector library which contained different promoters, ribosome binding sites and various fusion partners, we indentified factors which allowed us to obtain high amounts of soluble RI only in fusion with a MBP tag [8]. A fed-batch process was developed with the most favourable vector yielding about 800 mg of MBP-RI fusion protein per litre of mineral salt medium, which corresponds to 425 mg of RI. A similar result were recently published by Guo et al. [9]. The authors, in agreement with our earlier results, found the MBP tag the most suitable partner for soluble RI accumulation [9]. A drawback of this *fusion however is that the inhibitory activity of the MBP*-RI fusion *towards RNaseA is* 12-fold decreased compared to untagged RI [8]. However, despite high level production, all other cytoplasmic constructs containing untagged RI or RI fused to GST, SUMO and thioredoxin (TRX) showed high aggregation levels, independently from the transcriptional or translational control units which were varied in the constructs [8]. Based on these results we suspected that the above mentioned molecular features of RI - their sensitivity to the redox environment and hydrophobicity, or a combination of both, could be the main causes stipulating aggregation in the *E. coli* cells.

In this work we applied RI production strategies which were more focused on gaining knowledge and understanding about the significance of reduced SH groups for RI folding and its activity in *E. coli* cells. To manipulate RI folding we used the classical *in vivo* approach, which is based on supplementation of the cultivation medium with low molecular weight SH group acting materials.

So far, to our knowlege, all cases targeting on the improvement of the folding of recombinant proteins with folding aiding medium additives were performed with the aim to improve disulfide bond formation. Partially SH-group modifying agents were applied in combination with other stabilising agents. For example reduced/oxidized glutathione (GSH/GSSG) and arginine can easily penetrate the outer membrane and act in the periplasmic space on the folding of disulfide bond containing recombinant proteins [10](for review see [11]). Analogically, but more sophisticated *in vivo* folding approaches in the periplasmic space were based on the utilization of low molecular additives in tandem with co-secreted chaperones [12], or overexpression of the prokaryotic disulfide oxidoreductase DsbA [13], or disulfide isomerases, such as DsbC or eukaryotic protein disulfide isomerase (PDI) [14].

In difference to the periplasmic space, the cytoplasm is considered to be reduced and thus should be the preferred compartment for expressing a protein which contrains reduced cysteins. Externally added components can also affect the cytoplasm, as was reported by Gill et al. [15]. Folding and activtiy of chloramphenicol acetyltransferase (CAT) in the cytoplasmic space was altered due the presence of dithiothreitol (DTT) in the cultivation medium, i.e. DTT is also applicable to influence the redox state in the cytoplasm and consequently may be applicable for folding control in the cytoplasm.

By taking these earlier folding cases into account, and also considering the RI structural aspects, our intention was to generate and control a favourable redox situation for RI folding in the cytoplasmic and periplasmic compartments by applying reduced glutathione which is acting in the periplasmic space, and respectively, membrane permeable DTT which is acting in both compartments. This is highly interesting, as so far all studies only improved the redox conditions during periplasmic production by using the above mentioned methodologies. Also, all approaches aimed for disulfide production rather than keeping cysteins in a reduced state. Here, to our knowledge, for the first time we show the efficiency of this approach also for the production of proteins which need a strongly reducing environment. Surprisingly this approach worked well not only for periplasmic production, but also was necessary and working for cytoplasmic production. Aside from showing the feasibility of this approach at the example of an RI we go one step further and demonstrate that this approach is well suited also as a production strategy in typical fed-batch processes.

Results

The aim of the study was to produce correctly folded RNase Inhibitor (RI) in *E. coli*. We aimed to test whether it would be possible to produce RI as an authentic active protein (without any fusion) in the periplasmic or cytoplasmic compartments. We presumed that it would be possible to control the conditions in the periplasmic and cytoplasmic space by either process parameters or chemical additives.

Library screening in 96 well plates

In order to evaluate the best conditions for secretion of RI to the periplasmic space of *E. coli*, the RI gene was cloned by Gateway cloning into a periplasmic expression library which was earlier described [16]. This library contains a set of 36 different ColE1-derived plasmid vectors being a full factorial combination of three varying parameters: each three different IPTG inducible promoters and ribosome binding sites of different strength, and four well known signal peptides for translocation of the product protein into the peripasmic space. The RI gene harboring set of plasmids was transformed to the *E. coli* K-12 strain RV308 which additionally contained the plasmid pLT1 based reporter system for the monitoring of periplasmic folding stress by a *degP* promoter controlled luciferase cassette [16]. The σ^{24} dependent *degP* promoter is induced by periplasmic folding stress, i.e. if the protein of interest would aggregate during the accumulation in the periplasmic space.

As in our earlier study the initial screening experiments were performed in 96-well plates. For obtaining (i) well controlled conditions, (ii) enough cell material, i. e. high cell densities, and (iii) additionally applying strategies which would be applicable in the fermentation scale later, again, we were applying the EnBase cultivation technique in MWPs with pure mineral salt medium and starch-derived glucose as carbon source (cf. [8,17]). The EnBase-gel containing MWPs were directly inoculated with glycerol stocks of all 36 strains. Periplasmic RI synthesis was induced after 12 hours of cultivation by IPTG (Figure 1), the temperature was decreased to 22°C and 5 h later the cultures were harvested.

All constructs showed a good accumulation of RI. Remarkably, a 20-30% higher yield of RI was detected in the strains carrying vectors with the stronger promoters (pCU, pCTU) in combination with the strongest ribosome binding site (T7)(gels images not shown). However, unexpextedly, all samples showed a very low activity, independently on the expression strength or signal peptide (gels images and graphs not shown). These results would suggest that RI is expressed, but accumulated in inclusion bodies in all cases. However, intrerestingly this was not reflected by the luminescence signals

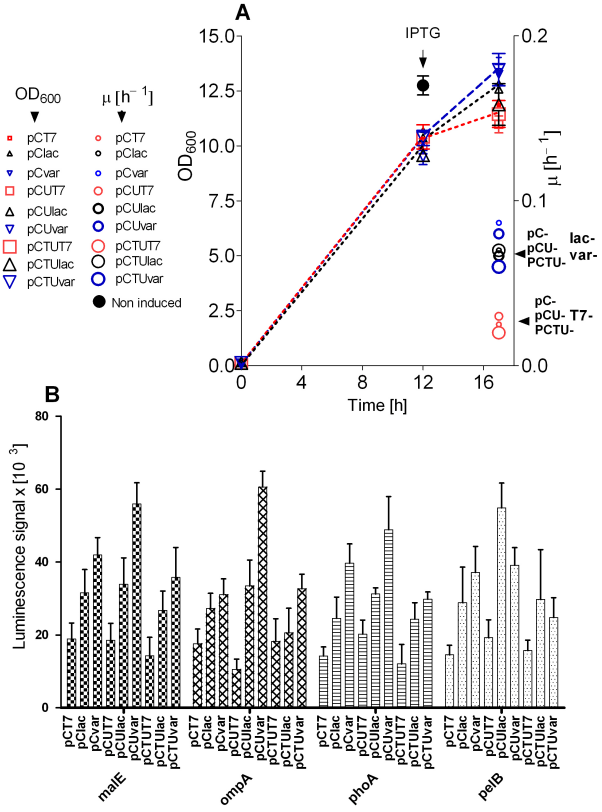

Figure 1 Growth with EnBase in MWPs of a library of 36 constructs with periplasmic RI production and luciferase activity of the different clones. A: OD_{600}'s are shown for all cultures of the vector library. Label sizes represent promoter strength [from weakest (pC, smallest labels) to strongest (pCTU, largest labels)]. Label shapes and colours represent ribosome binding strengths: T7 (red squares), lac (black triangle), var (blue triangle). Sizes and colours of the specific growth rate labels (circles) also indicate promoter and ribosome binding site strengths. **B**: The periplasmic aggregation signal measured as luminescence in samples of the RI expression library 5 h after induction. The data represent three independent cultivation experiments of the whole library.

in the different strains (cf. Figure 1). High luminescence signals were not connected to high RI accumulation levels (i.e. aggregated RI), but opposite - higher luminescence values were generated in the strains with the weaker promoters (pCU, pC) in combination with the weaker ribosome binding sites (var, lac). Therefore we consider for RI the periplasmic folding stress reporter system is not applicable to identify conditions for soluble periplasmic RI accumulation. This is clearly different to the earlier used cytoplasmic monitoring system [8] and to the results by Kraft et al. [16] with the scFv-miniantibody-phosphatase fusion and 11-β-hydroxysteroid dehydrogenase type 2 which were expressed in the same periplasmic expression library.

It may be worth to mention that the culture growth after induction of RI is dependent on the strength of product synthesis. The cell growth after RI induction of the clones harboring vectors with the higher synthesis rate stipulating elements, i.e. pCU, PCTU and T7, was strongly inhibited compared to cultures harboring vectors with weaker expression elements (pC, pCU, lac, var) which continued to grow (Figure 1).

Addition of reducing compounds

In summary, the first set of experiments showed that RI is well produced with some of the constructs, but that the product is neither active nor soluble. One major reason for the aggregation during recombinant production in *E. coli* could be the inability of the expression host to stably maintain the SH groups of the target RI. Therefore, we decided to supplement the cultivation medium with low molecular weight SH group stabilizing agents, such as DTT and reduced glutathione (GSH). Both are known to easily access the periplasmic space. Additionally, in parallel, target RI sensitivity to oxidation during the periplasmic accumulation was tested with and without reducing agents in an isogenic *dsbA* knockout mutant of the RV308 strain.

All these second round experiments were performed in shake flasks, with only a part of the set of plasmid constructs. The study was continued with the clones which during the screening in 96-well microwell plates resulted in the highest (pCUvar with *malE*, *ompA*, *phoA* signal peptides; and the pCUlac promoter with the *pelB* signal peptide - referred as the "first group") and the lowest luminescence levels (pCTUT7- *malE*, *ompA*, *phoA*, *pelB* - referred as the "second group"), respectively.

Cultivations were performed in glucose-MSM as described in the Material and Methods part. At the time of induction the cultivation medium was supplemented with the reducing agents DTT or GSH, respectively, in different concentrations. The results showed no effect on RI activity or improvement of RI accumulation in the soluble fraction in the cultures with 20 or 50 mM of GSH (graphs not shown). Only DTT resulted in a significant improvement of RI accumulation in the soluble fraction and also in an increased RI activity in both, *dsbA*$^-$ and *dsbA*$^+$, strains (Figures 2, 3). SDS-PAGE analysis of protein fractions from cultures with addition of DTT revealed an increasing intensity of 2 bands; their molecular sizes were corresponding to RI with a signal sequence (53 kDa) and without (50 kDa). The identity of the processed RI (50 kDa protein) was confirmed by N-terminal sequencing of 6 amino acids.

SDS-PAGE analysis showed that the highest amounts of unprocessed (app. 28 mg gCDW^{-1}) and processed RI (app. 12 mg gCDW^{-1}) were obtained in the RV308 *dsbA*$^+$ strain in the soluble protein fraction when the cultivation was performed with the pCUlac-*pelB*-RI vector with 12 and 18 mM of DTT. Under these conditions the yield of processed and even of unprocessed soluble RI protein was improved by 2 to 2.5-fold compared to the control without DTT (Figure 2). Different amounts of DTT did not affect the total amount of accumulated RI in the *dsbA*$^+$ strain, but both, DTT and GSH, had a highly negative impact on the growth and RI yield in the *dsbA*$^-$ strain.

After periplasmic production without DTT in the medium, RI activity was detected only in the *dsbA*$^-$ strains (Figure 4). Depending on the DTT concentration in the medium, total (soluble and insoluble) RI amounts in the *dsbA*$^-$ strain constructs were 2 to 4-fold lower compared to the controls without reducing agents (Figure 3). The negative effect of DTT on the accumulation of RI resulted also in a lower activity; cultures with 18 mM of DTT showed a 2 to 3-fold lower activity compared to the cultures with 12 mM DTT (Figure 4).

Despite their bad DTT tolerance, the yield of processed RI was improved in all *dsbA*$^-$ constructs when a lower DTT concentration was used (6 and 12 mM). In the *dsbA*$^-$ strains the highest yields of processed and soluble RI (app. 7 mg gCDW^{-1}) were obtained with the pCUvar-*malE* construct. In the other constructs the yield was 10 to 20% lower (Figure 2). In the *dsbA*$^-$ strains the yield of unprocessed RI was very similar for all constructs, but indeed 2 to 4-fold lower compared to the *dsbA*$^+$ strain. Also, the amounts of processed RI were highest in the *dsbA*$^+$ strain (30% higher than in the *dsbA*$^-$ strain) (Figures 2, 3).

The analysis of the insoluble protein fraction showed that after RI production with 6 to 12 mM DTT processed RI appeared in the insoluble protein fractions of both, *dsbA*$^+$ and *dsbA*$^-$ strains (Figures 2, 3).

Despite the significantly higher amounts of soluble RI achieved in the *dsbA*$^+$ strain, RI activities in total protein fractions of the *dsbA*$^-$ strain were just 10% lower. Interestingly, if the analysis was performed with the periplasmic fractions only, the *dsbA*$^-$ strain constructs showed even a 1.5 to 2-fold higher RI activity in comparison to the *dsbA*$^+$ strain (6 and 12 mM DTT, Figure 4).

The results of these experiments indicate that 12 mM of DTT is optimal for RI production in *dsbA*$^-$ and *dsbA*$^+$ strains. In contrast, GSH was not efficient for RI periplasmic accumulation. Thus it was not used in the further experiments.

Analogous experiments with the second construct group with the strongest promoters and ribosome binding sites (pCTUT7 constructs) also showed that the addition of 12 mM DTT to the medium was optimal for the RI production. However, compared to the first constructs, this second group resulted in a 4 to 5-fold lower yield of active and soluble RI. Remarkably, no RI activity

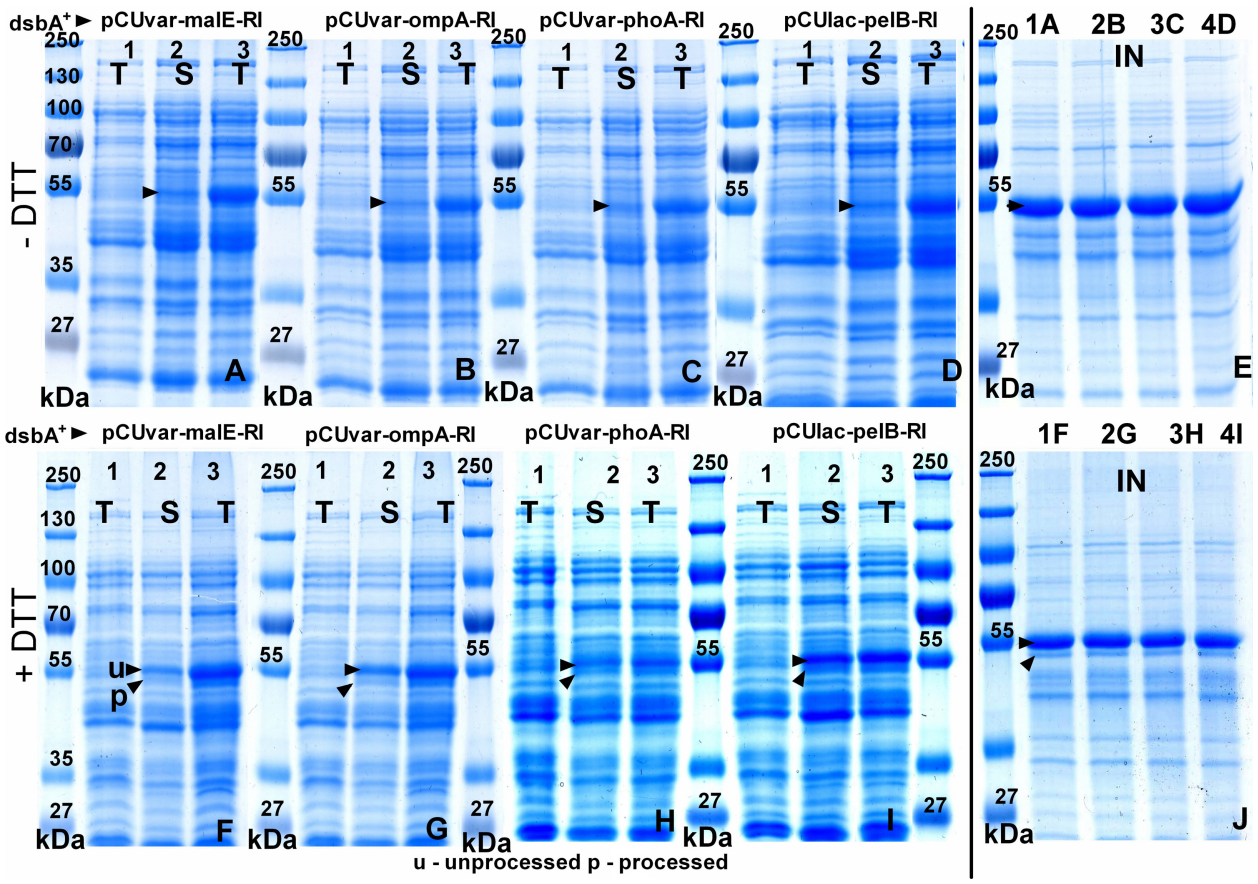

Figure 2 SDS-PAGE images of total (T), soluble (S), or insoluble (IN) protein fractions of samples from shake flask cultures of *E. coli* RV308 *dsbA*$^+$ with the following periplasmic constructs: pCUvar-malE-RI (A, F), pCUvar-ompA-RI (B, G), pCUvar-phoA-RI (C, H) and pCUlac-*pelB*-RI (D, I) 4 h after induction. Gels represent protein fractions after RI production without DTT (A-E) or with 12 mM DTT (F-J). Gels E and J represent insoluble protein fractions of the constructs without DTT (E) or with 12 mM DTT (J). Numbered lanes: 1 - total protein fraction 10 min before induction, 2 and 3 - soluble and total protein fractions 4 h after induction. Numbered lanes in gels E and J represent the insoluble fractions with the plasmids pCUvar-malE-RI (1A, 1F), pCUvar-*ompA*-RI (2B, 2G), pCUvar-*phoA*-RI (3C, 3H) and pCUlac-*pelB*-RI (4D, 4I). Protein size marker: PageRuler™ Protein Ladder Plus (Fermentas). For growth conditions see Materials and Methods. The amounts of applied protein are normalised.

was found in the periplasmic protein fractions of the *dsbA*$^+$ and *dsbA*$^-$ strains of this second group of constructs (graphs not shown).

Cytoplasmic RI production with DTT

Based on the results with the periplasmic expression vectors, which (i) clearly showed a positive effect of DTT not only on the processed form of RI but also for the unprocessed, i.e. signal peptide contaning form, we expected that the same approach also should improve the yield of cytoplasmically expressed RI. To evaluate this we selected the *E. coli* RV308 pCUlac-His6-RI construct which was earlier constructed (see [8]). In this construct a 6× histidine tag is fused to the N-terminus of RI. Otherwise this cytoplasmic construct is simlar to the periplasmic production construct pCUlac-*pelB*-RI.

The experiments for cytoplasmic RI production were performed analogously to the periplasmic expression with addition of different amounts of DTT at the time of induction. Here, clearly the RI activtiy was affected by the synthesis temperature and the DTT concentration (Figure 5). In more detail, DTT did not affect the yield of soluble RI if the production was performed at 37°C with all tested concentrations of DTT. Furthermore, 2 mM of DTT did not affect protein accumulation and activity of RI in the soluble fraction, independently from the synthesis temperature (Figure 5).

An obvious positive effect of DTT on the accumulation and activity of RI in the soluble fraction was detected if the production was performed at 30 or 22°C with at least 6 mM DTT. RI activities were increased by app. 30% compared to the controls without DTT.

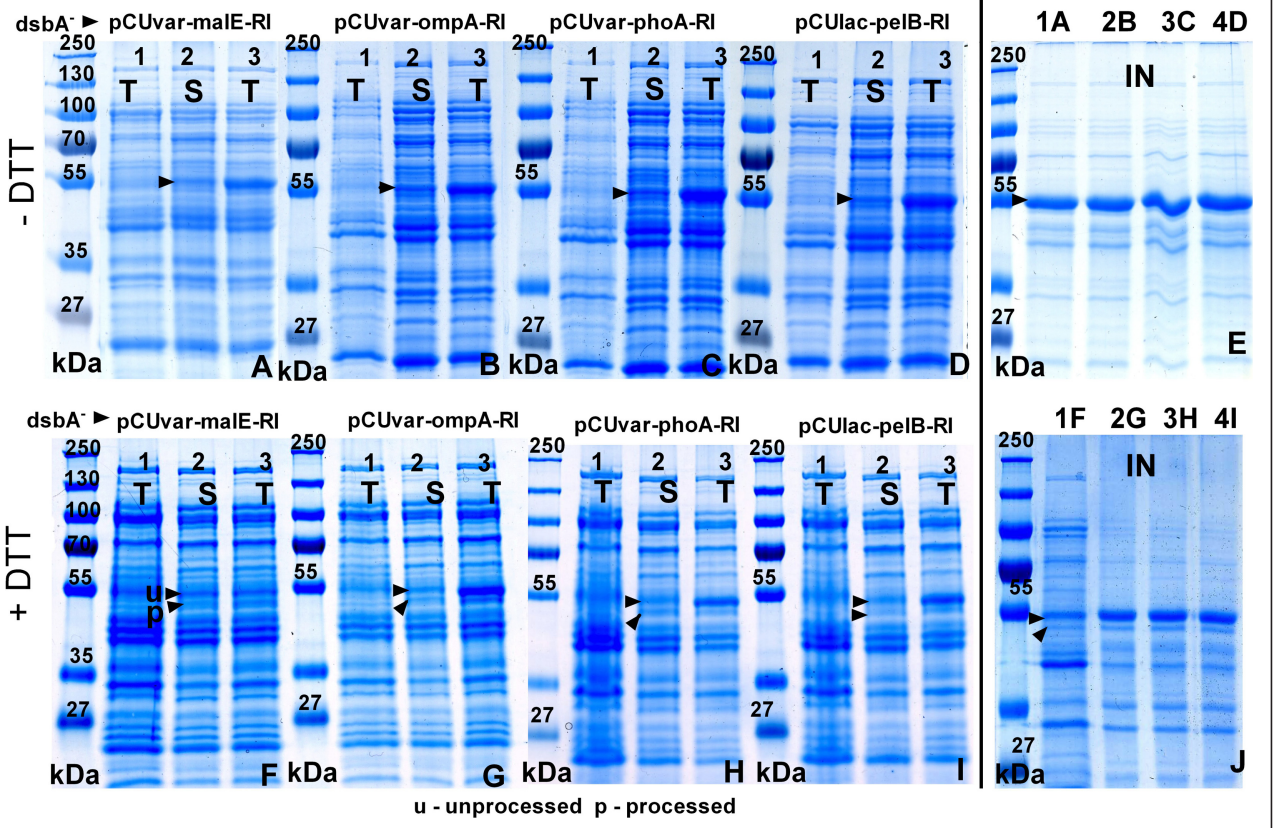

Figure 3 SDS-PAGE images of total cell extracts (T), soluble (S), or insoluble (IN) protein fractions from periplasmic production constructs of *E. coli* RV308 *dsbA*⁻. For vectors and conditions see Fig. 2.

A slight improvement of the RI activity was also observed even after production at 37°C with 12 and 18 mM of DTT (Figure 5). Indeed, the best results for cytoplasmic production were achieved after synthesis at 22°C with 12 or 18 mM of DTT in the medium, corresponding to ≈36 mg $gCDW^{-1}$ and ≈620 kU $gCWW^{-1}$ respectively (Figure 5).

Interestingly, if the yields between cytoplasmic and periplasmic cultures are compared, it is remarkable that the amounts of soluble RI were highly similar to cytoplasmic yields in the best periplasmic production constructs. Surprisingly, the RI actvity was even 3-fold higher with the cytoplasmic systems under comparable conditions.

RI production in fed-batch shake flasks with EnBase

After the optimal conditions for periplasmic and cytoplasmic RI production were defined, the next challenge was to test the effect of DTT on product accumulation and folding in the bioreactor under fed-batch conditions. Prior to the fed-batch bioreactor experiments the RI periplasmic and cytoplasmic production with 12 mM DTT in the medium were evaluated under fed-batch conditions in shake flasks by applying the EnBase technology. The experiments were performed with the following constructs: (i) for the periplasmic production the highest amount of processed and active RI yielding constructs: RV308 *dsbA*⁺ pCUlac-*pelB* and RV308 *dsbA*⁻ pCUvar-*malE*, (ii) for cytoplasmic production RV308 pCUlac-His6-RI.

All cultures performed with the *dsbA*⁺ strain showed a 2.6-fold improved RI production under the substrate limited condition, in both, the periplasmic and cytoplasmic constructs. However, in contrast to the earlier performed batch cultures, fed-batch production resulted in a 15 to 20% lower amount of active RI per cell unit (Figure 6). Under substrate limited conditions the *dsbA*⁻ strain grew very poorly and no product could be detected. Therefore, aside from the cytoplasmic constructs, only the periplasmic expression construct RV308(*dsbA*⁺) pCUvar-*malE* was used in the following bioreactor experiments.

RI production in bioreactors

Finally, batch and fed-batch periplasmic and cytoplasmic production of RI was performed in a stirred bioreactor.

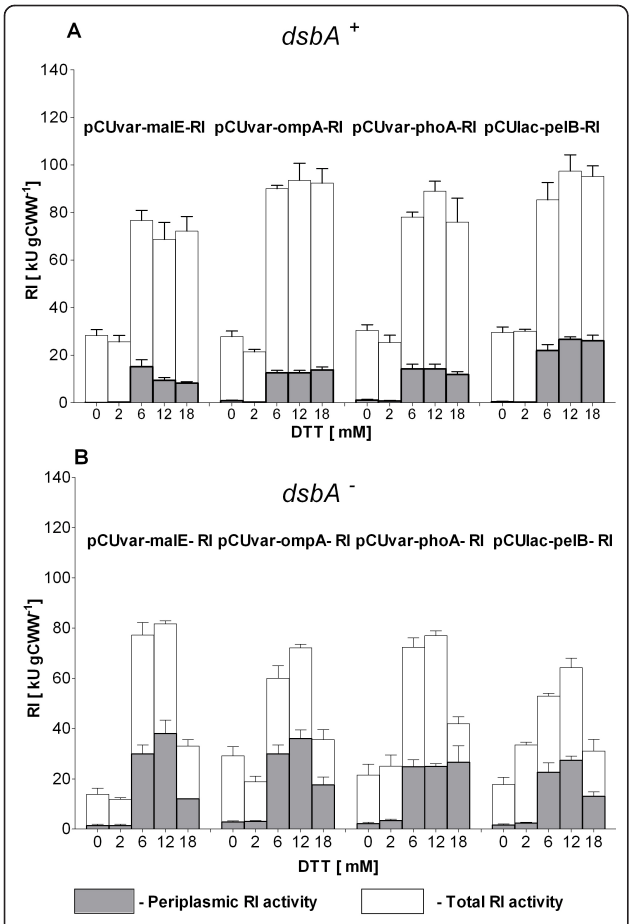

Figure 4 RI protein activities in total soluble and periplasmic fractions after 4 h of batch RI production in different *E. coli* RV308 *dsbA*$^+$ (A) and *dsbA*$^-$ (B) constructs at 22°C with 0, 2, 6, 12 or 18 mM of DTT. Culture conditions: glucose-MSM medium, induction with 0.2 mM IPTG, preinduction temperature 37°C, shift to the respective temperature at the time of induction.

In batch cultivations induction was performed at an OD_{600} of 7 ($\mu \approx 0.45\ h^{-1}$). In all fed-batch processes RI production was induced during the glucose-limited growth phase with exponential feeding at an OD_{600} of app. 28 ($\mu \approx 0.22\ h^{-1}$). After induction the feeding rate was further increased according to the predetermined feed function with the same μ_{set} as before induction. Also DTT was added at the time of induction as in the earlier experiments and concomitantly the temperatre was decreased from 37 to 22°C (Figure 7).

DTT is inactivated during bioreactor cultivation with a faster rate compared to shake flask cultures where the DTT oxidation rate was very low (Figure 8). After 4 hours of RI production in shake flasks only 5 to 10% of DTT was oxidized. In contrast more than 50% of DTT were oxidized during bioreactor cultivation already after 3 h. Therefore different approaches were tested and compared to keep the reduced state in the culture: (i) Single pulse addition of DTT to a final concentration of 12 mM at the induction point, (ii) single pulse addition of DTT to a final concentration of 12 mM 2 h after RI induction, and (iii) repeated (3 times) addition of DTT, starting at 2 h after induction (Figure 7).

Additionally, in order to prevent rapid DTT oxidization in all processes at the DTT addition point, the air flow was reduced from 30 L min^{-1} to 2 to 3 L min^{-1} to maintain the oxygen concentration in the medium close to zero. The reduction of the air flow was a necessary condition for accumulation of active product. Only at an reduced air flow rate the additon of DTT provoked a clear positive effect with highest yields, and interestingly this worked for both, the periplasmic and the cytoplasmic expression systems. However, despite a 30-fold higher volumetric productivity in the fed-batch bioreactor processes compared to the analogous batch processes, the periplasmic and cytoplasmic RI yields per cell remained 10 to 15% lower in the fed-batch cultivations with the same DTT addition mode (Figure 9).

Protein activity analysis in the crude extracts of samples from the fed-batch cultures revealed - different from the shake flasks - that RI activity was only improved by 1.4-fold by single DTT addition, but 2 to 3-fold when the repeated DTT addition approach was applied (Figure 9). This is not surprising, because even the minimum air flow, maintaining 0% of oxygen concentration in the medium, resulted in 60% of DTT oxidation during the whole bioreactor process (Figure 8).

Discussion

In this work, for the first time, low molecular weight SH group modifying agents were utilized for periplasmic and cytoplasmic folding improvement of a cysteine-rich LRR model protein with exclusively reduced cysteins, a recombinant ribonuclease inhibitor (RI). We demonstrated the effect of SH group modifying agents on periplasmic and cytoplasmic activities of RI and its soluble accumulation. We also showed that the reason for the non-successful approaches for expression of active RI in *E. coli* is its incapability to create an optimal redox environment for RI folding even in the cytoplasm. In addition, obviously RI folding *in vivo* does not just depend on the redox conditions. Only the combination of lower translation rate, low post-induction temperature and strongly reducing conditions resulted in a reasonable yield of RI and these conditions could be applied for periplasmic as well as for cytoplasmic RI production. Furthermore, on the basis of these principles, which were evaluated in small-scale cultures, production of RI was also successful during fed-batch cultivation in a stirred tank bioreactor.

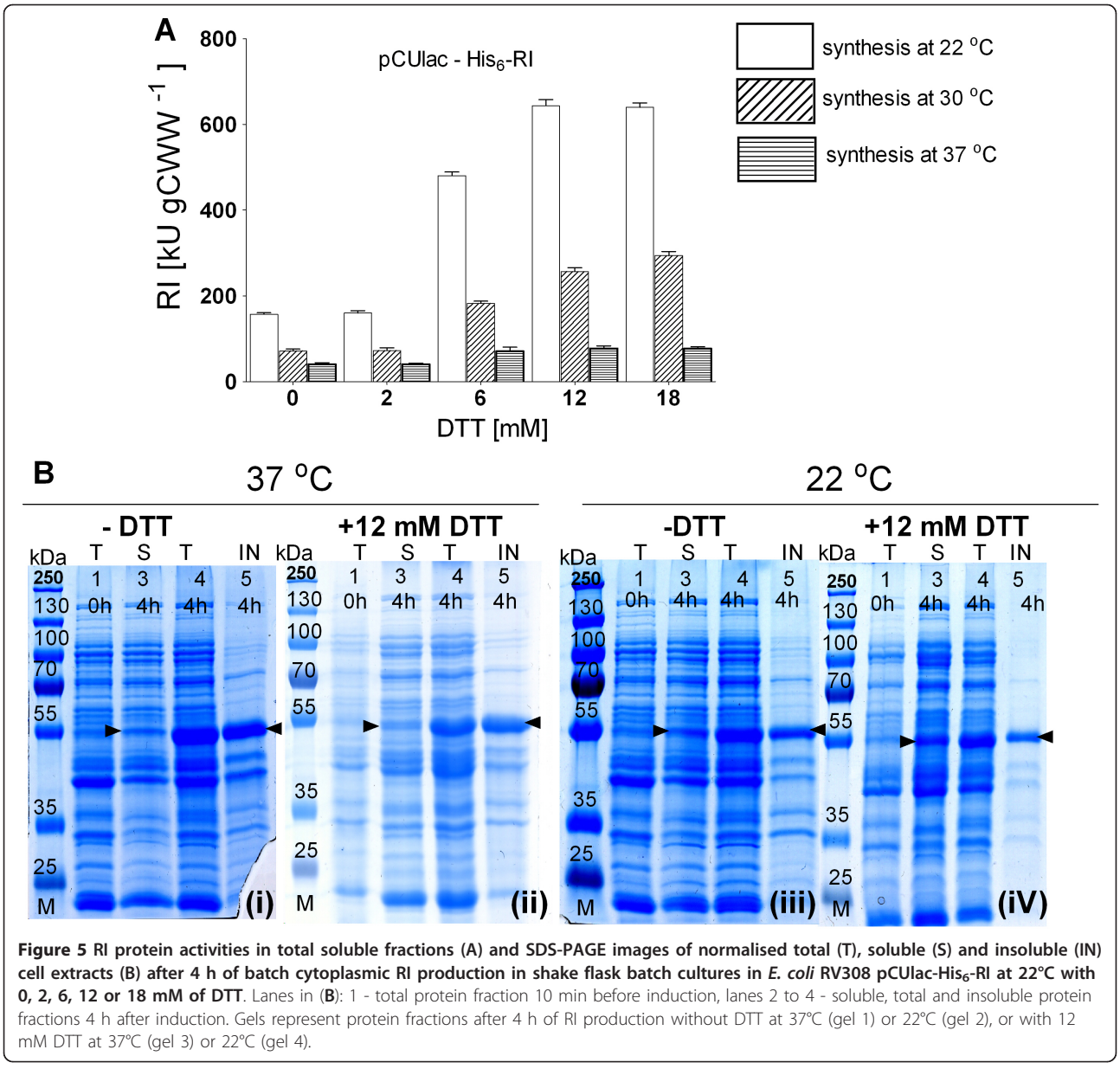

Figure 5 RI protein activities in total soluble fractions (A) and SDS-PAGE images of normalised total (T), soluble (S) and insoluble (IN) cell extracts (B) after 4 h of batch cytoplasmic RI production in shake flask batch cultures in *E. coli* RV308 pCUlac-His$_6$-RI at 22°C with 0, 2, 6, 12 or 18 mM of DTT. Lanes in (**B**): 1 - total protein fraction 10 min before induction, lanes 2 to 4 - soluble, total and insoluble protein fractions 4 h after induction. Gels represent protein fractions after 4 h of RI production without DTT at 37°C (gel 1) or 22°C (gel 2), or with 12 mM DTT at 37°C (gel 3) or 22°C (gel 4).

Screening of the best constructs for soluble production of RI

In the first part of the work, for evaluation of significance of keeping reduced conditions, RI folding was compared by expressing RI in the periplasmic compartment of the *E. coli* K-12 strain RV308 and its isogenic *dsbA*$^-$ mutant. This approach was based on the well known fact that the redox status in the periplasm could be easily controlled by the addition of SH group stabilizing agents, such as DTT and GSH, to the cultivation medium. However, previous approaches mostly aimed for optimising the redox conditions for disulfide bond isomerisation (e.g. [12-16]). As a first step, however, we tested whether different combinations of promoter, ribosome binding site and signal sequences stipulate soluble RI accumulation in periplasmic space as a kind of initial standard conditions. Therefore we applied a previously published plasmid library for periplasmic production together with a periplasmic folding reporter system [16]. The periplasmic RI folding in all production constructs was evaluated by using the experimental set up published recently by Šiurkus et al. [8], combining fed-batch RI production in MWPs with luminescence based high throughput screening. Interestingly, and in difference to the earlier published results with the screening for soluble human epidermal growth factor receptor and human 11β-hydroxysteroid dehydrogenase type 2, in case of RI the screening results did not match

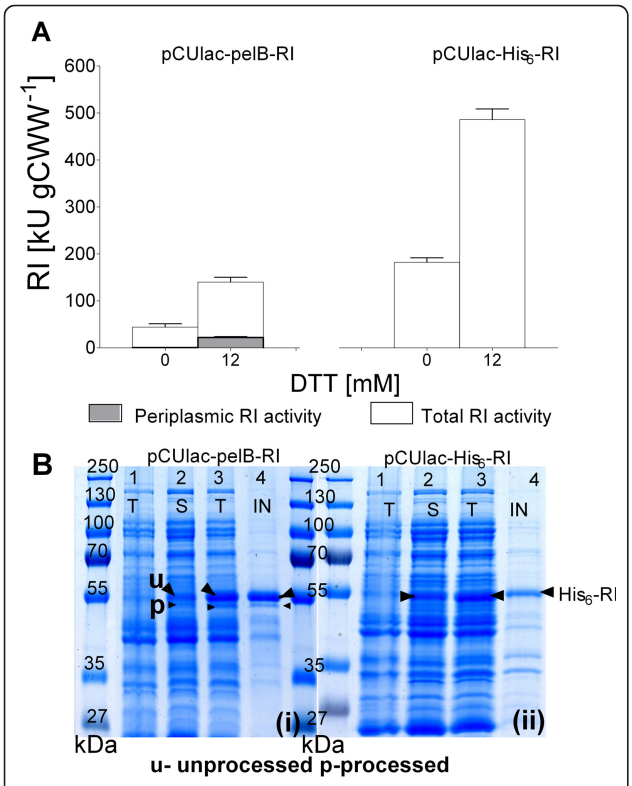

Figure 6 RI protein activities in total soluble and periplasmic fractions [in kU (gCWW)$^{-1}$] of *E. coli* RV308 pCUlac-pelB-RI and *E. coli* RV308 pCUlac-His$_6$-RI after EnBase cultivation in shake flasks with RI induction for 4 h at 22°C without or with 12 mM DTT (A), and SDS-PAGE images of total (T), soluble (S) and insoluble (IN) protein extracts (normalised to equal amounts protein) (B). Left gel: *E. coli* RV308 dsbA$^+$ pCUlac-*pelB*-RI (periplasmic expression construct); right gel: *E. coli* RV308 pCUlac-His$_6$-RI (cytoplasmic production construct). Lanes: 1 - total protein fraction 10 min before induction, 2 to 4 - soluble, total, and insoluble protein fractions 4 h after induction with 12 mM of DTT. Protein size marker: PageRuler™ Protein Ladder Plus (Fermentas). Data originate from three experiments.

with the soluble product amount in the periplasmic space. In our case the weaker expression elements harbouring periplasmic production constructs gave higher luminescence signals compared to the strong pCTUT7 constructs. We suggest that in case of very poor product solubility, and thus high luminescence signals already in the weaker expression vectors, lower luminescence in the stronger expression vectors is a result of lower luciferase production by an overloaded cellular production machinery. Accordingly, the strongest growth inhibitory effect was observed for the pCTUT7 constructs in which the expression of luciferase was lowest. Weaker expression stipulating constructs (promoters pC-, pCU-, RBS: -lac, -var) resulted in high luminescence signals during periplasmic RI production because a part of the product was found in periplasmic inclusion bodies on one side, but on the other side the weaker target protein expression in these vectors did not consume all cellular recourses needed for luciferase production and cellular growth. Thus in summary, luciferase signals in combination with the periplasmic library have to be evaluated with caution.

As the initial screening results provided no clear answer on the preferable constructs, both, a strong and a weaker expression construct were selected for the further studies. As the previous results [8] indicated aggregation in the cytoplasm as a major problem, the following work was focussed on the control of the redox conditions. This was parallel approached by using a *dsbA*$^-$ mutant to remove of the strong oxidising activity of DsbA, and in parallel applying reducing agents. Interestingly, in our case GSH was not active, neither in the *dsbA*$^+$ nor in the *dsbA*$^-$ mutants. However, in contrast DTT worked well. More favourable conditions for RI folding in the periplasmic space were created in the *dsbA*$^-$ mutant, but this mutant seemed also to be highly sensitive to DTT and could not be productive in the presence of higher concentrations than 6 mM DTT. By considering all results of the separate analysis of the amounts of processed (i.e. without signal peptide) and non-processed forms of RI (i.e. with signal peptide), we conclude that RI accumulation in the periplasmic space was improved due to the primary effect of DTT on the cytoplasm, where it avoided aggregation of the protein, even in the case of cytoplasmic RI expression vectors.

The significance of the synthesis rate of RI for its periplasmic accumulation and folding was clearly demonstrated from RI periplasmic production experiments with DTT in the strong and weak expression stipulating constructs groups. The results showed that DTT was highly effective only in weaker expression rate stipulating constructs, the balance between the synthesis and the folding rate is important for obtaining soluble product. In contrast DTT was not effective for RI folding in the strong expression elements harbouring constructs in which obviously the RI synthesis rates were too high.

The leader peptide had a lower impact on the accumulation of processed RI. For further studies the *pelB* leader peptide was selected as the most suitable for RI periplasmic accumulation in dsbA$^+$ strain. However, also the *ompA*, *malE* and *phoA* leader peptides stipulated the periplasmic accumulation of RI in the presence of DTT.

Our results are in good agreement with other periplasmic production cases. For example the significance of synthesis rate on periplasmic accumulation and aggregation of recombinant penicillin G acylase was also demonstrated by Sriubolmas et al. [18]. The authors showed that the cytoplasmic and periplasmic aggregation of penicillin G acylase depend on the synthesis rate, which was altered by varying the amounts of IPTG.

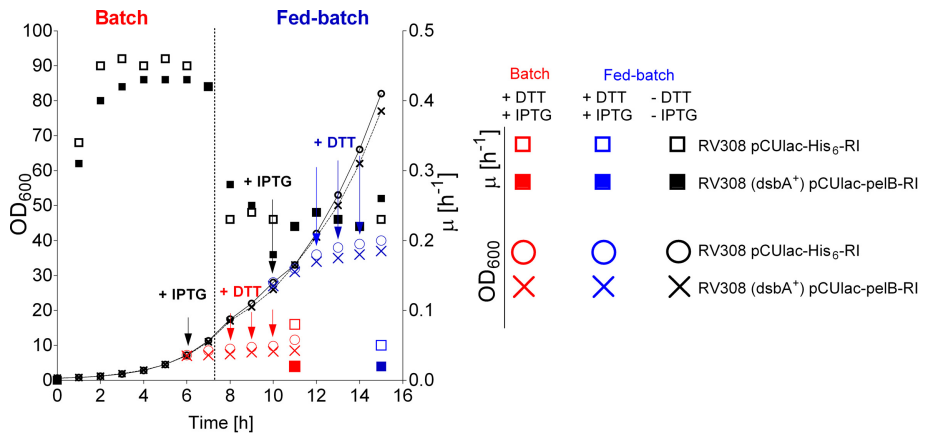

Figure 7 Growth curves of *E. coli* RV308 pCUlac-*pelB*-RI (periplasmic expression construct) and *E. coli* RV308 pCUlac-His$_6$-RI (cytoplasmic production construct) without (control) and with RI production in a batch and a fed-batch process with exponential glucose feeding in a 10 L bioreactor. For conditions see Material and Methods. Black symbols: control culture without induction; red symbols: batch bioreactor cultivation, blue symbols: fed-batch bioreactor cultivation.

In addition, RI accumulation patterns, obtained after RI synthesis with DTT, represented by premature and processed RI forms in the insoluble and soluble protein fractions, are typical for periplasmic production. Similar pattern were reported by Sriubolmas et al. [18] for penicillin G acylase and by Bowden et al. [19] for β-lactamase. In both cases a mixture of precursor polypeptides with signal peptides and processed periplasmic proenzyme forms was detected.

Finally, another interesting optimisation case may be mentioned, involving the same vector library and the periplasmic misfolding reporter. Soluble periplasmic accumulation of human 11β-hydroxysteroid dehydrogenase type 2 and scFv-miniantibody phosphatase was highly dependent on the leader peptide but not on the expression rate regulating genetic elements. Indeed, lower periplasmic aggregation levels and more efficient export to the periplasmic space were observed for the constructs with the weaker pCU promoter and the *lac* ribosome binding site, compared to the strong expression pCUT7 vectors. [16]

The effect of DTT on the RI cytoplasmic folding

The RI activity was clearly dependent on the DTT concentration in the medium. Additionally, besides DTT, cytoplasmic folding of RI strongly depended on the production temperature as a synthesis rate and folding regulating factor. Even when RI production was carried from a weaker promoter and ribosome binding site, the lower production temperature stipulated a better RI

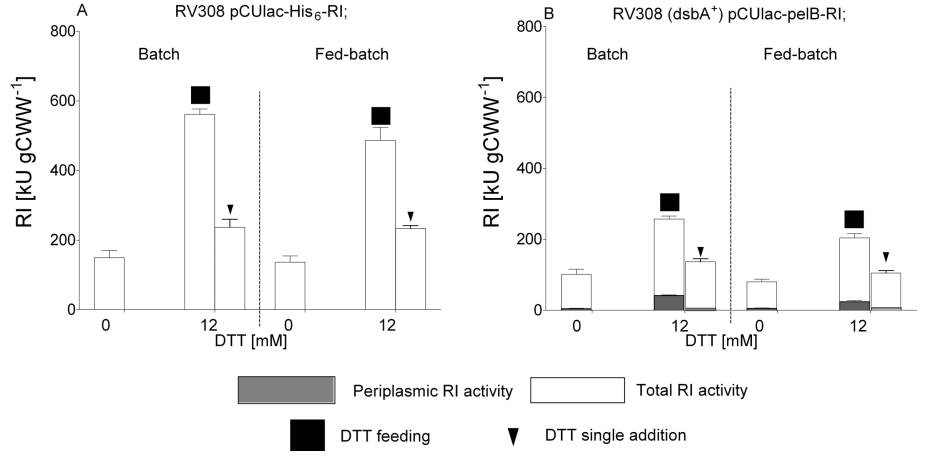

Figure 8 The amount of reduced DTT during batch shake flask and fed-batch bioreactor processes with microarerobic production mode, was measured by using Measure-iTTM Kit (Invitrogen). The samples for reduced DTT evaluation were taken every synthesis hour starting with DTT addition moment. The data is derived from three assays.

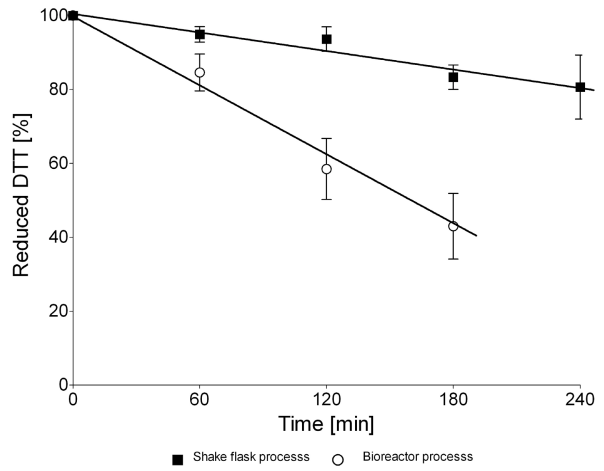

Figure 9 RI activities in the total soluble protein fraction (white bars) and in the periplasmic fraction (grey bars) of *E. coli* of RV308 pCUlac-*pelB*-RI and RV308 pCUlac-His_6-RI from samples of batch and fed-batch bioreactor cultivations without and with addition of DTT. Data derive from three activity assays.

folding. Compared to best periplasmic production results, the cytoplasmic production construct gave 3 to 4-fold higher total RI activity, although just 30% less premature RI was produced in the best periplasmic production construct. We suggest that the non-processed N-terminal signal sequence, which is 3-fold longer compared to the 6× His-tag can negatively affect the ability of RI to interact with Rnase E. A negative effect of an N-terminal tag on the activity of RI was also observed after RI production as a fusion with MBP where the RI specific activity was about 12-fold reduced (Šiurkus unpublished data).

In our opinion, the RI solubility was improved due to the complex DTT effect on (i) RI SH groups and (ii) reduced expression rate stipulated by DTT toxicity, genetic elements and lower production temperature. Without doubt DTT could also have a negative impact on the overall target protein yield due to the highly induced stress related proteins as reported by Han et al. [20]. Gill et al. [15] reported increased protease activities and heat shock protein synthesis in *E. coli* JM105 and RR1 strains after recombinant CAT production in a bioreactor due to the presence of relatively low amounts of DTT in the medium. Surprisingly our strain demonstrated a comparably high tolerance to DTT. It was still very productive in the medium containing a total DTT concentration of close to 20 mM which according to Missiakas et al. [21] should be a lethal for *E. coli*.

Interestingly, DTT served as an SH group modifier *in vivo* not just in the periplasmic space but also in the cytoplasm. It was obvious from the cytoplasmic production results that the redox environment in the *E. coli* cytosol is not optimal for a target protein with a high content of reduced cysteins. The *E. coli* cytoplasmic environment is in general reduced, but oxidative damage occurs when cells enter the stationary phase and starvation [22,23]. That would possibly lead to target protein SH group oxidation.

RI fed-batch production in shake flasks

Enbase experiments provided valuable information for the process development in the bioreactor. The *dsbA*$^-$ strain turned out to be inable to maintain its productivity under substrate limited feed conditions with DTT and thus would not be favorable for futher bioprocess development. In contrast the cytoplasmic and periplasmic fed-batch production patterns in the constructs with the *dsbA*$^+$ strain with 12 mM of DTT were similar to the batch shake flasks, showing that the substrate limited cultivation mode has no negative effect on the host productivity and protein folding in our case. Thus Enbase clearly helped to save time and labor in hte process develpment process.

Bioreactor processes

The bioreactor experiments showed that highly aerated cultivation medium could be a very oxidative environment. That should be considered when compounds which are sensitive to oxidation are used for protein folding, recombinant synthesis induction and/or plasmid stabilization. Microaerobic or fully anaerobic production strategies would preserve more oxidation sensitive chemicals. On the over hand respiration is a key factor for recombinant productivity. Anaerobic conditions generally result in poor growth and are often considered as unfavourable for recombinant protein production, by limited energy production, acidification of the cytoplasm by organic acids and the large synthetic requirements which are needed to establish the anaerobic responses [17,24,25]. Accordingly, we did not succeed to produce RI when the air flow was downregulated at the induction point, however, we could solve the problem by later addition of DTT and concomittant reduction of the airflow.

In our case DTT oxidation in the bioreactor was the main concern during development of the bioreactor process. We succeeded to prevent rapid DTT oxidation, which was obvious if the DO was maintained at approximately 30%, by strongly reducing the aeration. Thus we created conditions which are usually present in shake flasks [17]. After medium supplementation with DTT the air flow was not completely switched off, but down-regulated in order to maintain the actual oxygen concentration close to 0%.

This down-regulation of the air flow rate had a drastic effect on the cell productivity when it was performed at

the same time with DTT addition and RI synthesis induction. In this case no RI production was found in periplasmic and cytoplasmic constructs. In our opinion the combination of temperature reduction, induction and DTT addition stipulated a huge metabolic burden as earlier defined by Glick [26]. In order to reduce the stress, we decided to induce recombinant production separately from medium supplementation with DTT and down-regulation of the air flow. Thus only the temperature shift was performed at the time of induction, but the reducing agent was added only 2 hours after induction when the RI production reached its maximum, and at the same time the air flow was reduced.

Furthermore, unexpectedly, after single medium supplementation with DTT much lower RI activity per cell unit was detected in the bioreactor production process compared to the shake flasks. However, the total RI production level per cell unit in the bioreactor was similar to the shake flask results. We suspected that the decreased RI activity in the bioreactor could be related to the partial oxidation of the DTT even at the very low air flow rate, as confirmed by the analysis of the DTT oxidation rate. Whereas during RI production only 10% of the DTT was oxidized in shake flasks, 60% was found to be oxidised in the bioreactor even at microaerobic conditions. To counteract this we tested whether repeated DTT addition would improve the yield of active RI. As expected, repeated DTT addition strongly enhanced RI folding during batch and fed-batch processes. In this case the amount of active RI per cell unit in the bioreactor processes was similar to the activities which were detected in samples from shake flasks. The effective RI production with DTT under batch and fed-batch conditions shows that the *in vivo* approach for RI folding is reproducible, independently from the cultivation mode and cell densities.

Conclusions

In this study we demonstrate the successful production of active RI by periplasmic and cytoplasmic approaches based on the artificial control of the redox conditions and the expression rate via external manipulations with medium components and cultivation parameters. The folding approach presented here could be very useful for recombinant protein production not just distinguished by reduced SH groups but also for disulfide bond containing proteins. In our opinion the combination of oxidised/reduced DTT in tandem with the cellular oxidation/reduction machinery and cultivation parameters could enhance oxido-(re)shuffling needed for correct formation disulfide bonds in the cytoplasm. Our folding approach could be applied as an alternative for protein synthesis in the periplasmic compartment where the main synthesis bottleneck is protein transfer across the periplasmic membrane.

Methods

Vector library preparation

A previously described periplasmic expression library [16], containing 36 periplasmic expression vectors, was kindly provided from Hans-Knöll-Institute, Jena, Germany. The RI encoding gene was inserted into periplasmic expression vectors via site specific recombination reaction based on the Gateway® cloning technology (Invitrogen) as described by [8].

Preparation of target protein expression platforms

The expression strain *E. coli* K-12 RV308 (ATCC 31608) was first transformed by using the calcium temperature shock method with the periplasmic folding stress reporter plasmid plt1 previously described by Kraft et. al. [27], carrying a resistance for ampicillin, and plated on LB agar with ampicillin (100 μg mL^{-1}). RV308 plt1 was co-transformed with the library of 36 the RI gene containing periplasmic expression vectors. The transformants were plated on LB agar containing ampicillin (100 μg mL^{-1}) and chloramphenicol (30 μg mL^{-1}). The cell stock was produced after 8 h of recombinant strain cultivation in 10 mL of LB medium in 100 mL shake flasks at 37°C and 220 rpm. All culture suspensions with OD_{600} of 4 ± 0.2 were mixed with an equal volume of sterile 50% glycerol solution to achieve a final glycerol concentration of 25%. The glycerol culture suspensions were aliquoted into sterile PCR strips and stored at -70°C. For RI cytoplasmic expression experiments the previously described RI expression construct RV308/pCUlac-His_6-RI [8] was used.

Engineering of E. coli RV308 dsbA$^-$ strain

The *dsbA* gene in RV308 was in inactivated by P1 transduction. E. coli JW3832 from Keio collection was used as the donor for the *ΔdsbA::kan* marker. The RV308 clones harboring *ΔdsbA::kan* were selected after cultivation on solid LB medium containing kanamycin antibiotics. In addition, the mutation in selected clones was confirmed by PCR analysis, with the following forward and reverse primers: 5'-aagatttggctggcgctggct-3' and 5' - tcggacagatatttcactgtatca - 3. The strains - RV308 *dsbA*$^+$ and JW3832 *ΔdsbA::kan* were used as controls in the PCR analysis.

Cultivation media

Transformations and plasmid propagations were performed on solid and liquid LB medium containing Bacto-Tryptone (10 g L^{-1}), Bacto-yeast extract (5 g L^{-1}), NaCl (10 g L^{-1}), 15 g L^{-1} bacto agar (if solid medium) and the required antibiotics. Fed-batch and batch cultivations were performed in glucose-based mineral salt

medium (MSM) with the following composition (per litre): Na_2SO_4 2 g, $(NH_4)_2SO_4$ 2.68 g, NH_4Cl 0.5 g, $KHPO_4$ 14.6 g, $NaH_2PO_4 \times H_2O$ 3.6 g, $(NH_4)_2$-H-citrate 1.0 g, and glucose 2.5 to 15 g. NaOH (40%) was used to adjust pH to 7.0 prior heat sterilisation. Additionally, before cultivation on the mineral salt medium the following sterile solutions were added: 3 mL L^{-1} of (1 M) $MgSO_4$ and 2 mL L^{-1} of trace element solution with the following composition (per litre): $CaCl_2 \times 2H_2O$ 0.5 g, $ZnSO_4 \times 7H_2O$ 0.18 g, $MnSO_4 \times H_2O$ 0.1 g, Na_2-EDTA 20.1 g, $FeCl_3 \times 6H_2O$ 16.7 g, $CuSO_4 \times 5H_2O$ 0.16 g, $CoCl_2 \times 6H_2O$ 0.18 g; and thiamine hydrochloride (0.1 mM), ampicilin (100 mg L^{-1}) and chrolamphenicol (30 mg L^{-1}). The feeding solution for fed-batch cultivations was based on fully formulated MSM with the required antibiotics and 550 g L^{-1} of glucose.

Fed-batch mode cultures and recombinant protein synthesis in 96 microwell plates

Agar based EnBase® 96 microwell plates (MWPs) were purchased from BioSilta Oy Oulu (Finland). For cultivations in MWPs 10 μl of glycerol stock cultures were transferred to cultivation wells containing 150 μl of fully formulated MSM medium with 6 AGU L^{-1} of EnzI'm (BioSilta Oy). Periplasmic RI expression was induced after 10 hours of cultivation at 30°C at an OD_{600} of 12 ± 2 ($\mu \approx 0.16\ h^{-1}$) by addition of IPTG (0.2 mM final concentration). All microscale cultures were cultivated by intensive shaking with a Variomag® Thermoshake (Inheco, Germany), shaking diameter 1.5 mm, at 30°C and 750 rpm. After induction the temperature was decreased to 22°C and the cultures were harvested 5 h after induction. The OD_{600} measurements and the luminometric assay for assaying the target protein periplasmic misfolding levels in MWP's were performed as earlier described [8].

Batch mode cultivations in the shake flasks

The inoculums for batch protein production in shake flasks were prepared by overnight batch cultivation of the selected clones in 500 mL shake flaks with 50 ml of glucose-MSM with 10 g L^{-1} of glucose at 37°C. For protein production 5% of the corresponding inoculum culture was transferred to fresh glucose-MSM containing the same amount of glucose at a final volume of 200 mL in 1 L baffled Erlenmeyer flasks. Cultures were grown at 37°C and 180 rpm until they reached a cell density of OD_{600}= 1 ± 0.05 ($\mu \approx 0.35\ h^{-1}$) where induction was performed by addition of IPTG (final concentration 0.2 mM). DTT was added to the cultivation medium at the RI induction point as dry powder to achieve the needed concentration of 2 to18 mM, and reduced glutathione was added with a final concentration of 20 or 50 mM, respectively. The temperature was shifted at the time of induction to 22°C and the cultures were continued fro 4 h at the shaking rate of 180 rpm.

Fed-batch cultivations in shake flasks

The fed-batch shake flask cultivations were performed with the gel-based EnBase system in 1 L baffled Erlenmeyer flasks with 200 mL of MSM medium as earlier described [8].

Glucose release for substrate limited growth was generated by 12 AGU L^{-1} in the cultivation medium. Product synthesis in the selected expression platforms was induced at OD_{600} = 5 ± 0.5 ($\mu \approx 0.22\ h^{-1}$). Induction was performed by addition of IPTG to a final concentration of 0.2 mM. The necessary amount of DTT was added as dry powder to the cultivation medium to achieve a final concentration of 12 mM. After induction the cultures were continued for 4 h at 22°C at a shaking rate of 180 rpm.

Bioreactor processes

Batch and fed-batch cultivations were performed in a 10 L working volume Biostat C bioreactor (B. Braun Biotech, Melsungen, Germany) with the following parameters: the pO_2 was maintained at 30% by adapting the stirrer rate and automatic regulation of the air flow (from 0 to 30 liters per min), the cultivation temperature before RI induction was 37°C. After induction it was downregulated to 22°C and kept until the end of the process. The pH was controlled at 7.0 ± 0.1 by addition of NH_4OH (25%) or H_3PO_4 (2 M).

The feeding rate was controlled by the Biostat software (version 4.62). The process was monitored by the MFCS/win 2.0 supervisory system. Fed-batch cultivations were started with a volume of 8.0 L of MSM with 15 g L^{-1} of glucose. Exponential feeding profiles were programmed to maintain a specific growth rate of $\mu \approx 0.22\ h^{-1}$. The feeding profiles were calculated with following equations:

$$F(t) = F_o e^{\mu t}$$

where F_o is the initial feeding rate [L h^{-1}], μ is the specific growth rate [h^{-1}] to be maintained during feed operation, and t is the time after feed start [h]. The initial feeding rate was calculated from the mass balance on substrate according to

$$F_0 = \frac{\mu X_0 V_0}{S_f Y_{x/s}}.$$

Here, X_0 and V_0 are the cell dry weight (CDW) [g L^{-1}] and the culture volume [L] at the time of the feeding start, respectively, S_f [g L^{-1}] is the substrate concentration in the feeding solution, and $Y_{x/s}$ is the yield coefficient (g CDW per g of glucose). $Y_{x/s}$ in all cases

(cytoplasmic and periplasmic expression strains) was calculated from batch fermentations as 0.3 g g^{-1}.

Before initiation of the fed batch mode cells were cultivated as a batch until OD_{600} ≈ 18. RI synthesis was induced during the fed-batch cultivation mode at an OD_{600} of 28, the specific growth rate at the time of induction was the same in all cases (μ = 0.22 h^{-1}). The exponential feed function was continued after induction in all fed-batch experiments. Batch cultures were induced at an OD_{600} of 7 (μ ≈0.45 h^{-1}).

In all bioreactor synthesis experiments 148 mL of 0.65 M DTT solution were added after 2 h of RI induction to achieve a final concentration of 12 mM in the cultivation medium. In the experiments with DTT feeding the same solution was added repeatedly, starting at 2 hours after RI induction by addition of 148 mL of 0.65 M DTT solution and continued by repeated addition of 74 mL 0.65 DTT solution every following 60 min. At the first DTT addition point the air flow was decreased from 30 to 2-4 L min^{-1} and the stirrer was manually regulated to maintain 0% of oxygen concentration in the cultivation medium. In all cases the target protein synthesis continued for another 3 hours.

Analytical tools

Cell samples harvested from flask and bioreactor cultivations were resuspended in lysis buffer with the following biomass to buffer ratio: 1 g of biomass with 10 mL of lysis buffer (50 mM Tris-H_3PO_4 pH 8.0, 0.1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 15 mM DTT, 10% propyleneglycol and 0.1 mg mL^{-1} lysozyme). After 30 min of lysis at +4°C the biomass was sonicated for 60 sec (Vibra cell™, Sonic and Materials Inc., 6 mm diameter probe tip) at 4°C. The soluble and insoluble protein fractions were separated by centrifugation for 30 min, 14000 rpm, 4°C. The total protein fraction represents cellular debris suspension (crude extract) before centrifugation. After centrifugation the insoluble protein pellet was additionally washed and resuspended in the original volume of lysis buffer without lysozyme. The periplasmic protein fractions were extracted by the standard osmotic shock procedure. Therefore after centrifugation the cell pellet was resuspended in 5 mL of ice cold solution, containing 20% (w/v) sucrose, 100 mM Tris-H_3PO_4 (pH 8.0), and 0.5 mM Na_2EDTA. After incubation for a 10 min at +4°C cells were harvested by centrifugation at 10,000 rpm for 10 min and +4°C. After removal of the supernatant the cell pellet was again resuspended in 5 mL of ice cold deionised water, containing 15 mM of DTT. After another incubation for 10 min and centrifugation the supernatant (containing the target protein) was suplemented with 2.0 ml of buffer (250 mM Tris-H_3PO_4 pH 8.0, 0.4% Triton X-100, 8 mM EDTA, 4 mM PMSF, 30% propylene glycol).

Samples for SDS-PAGE separation were prepared as follows: 20 μL of protein sample (total soluble, insoluble, protein suspensions), 25 μL of 4×SDS-PAGE loading buffer (Fermentas), 5 μL of 20×DTT (Fermentas) and 50 μL of deionised water to obtain a final sample volume of 100 μL. Samples were heated for 15 min at 95°C. 10 μL of sample was applied to each lane of a 10% SDS-PAGE gel.

The amounts of target were determined from scanned SDS-PAGE gel images with TotalLab software. The gels with separated sample proteins were produced for TotalLab quantifications with internal BSA standards (3 concentration points).

The amount of active RI in the soluble fraction was determined by an activity assay described by Blackburn et al. [28] and is presented in kilo units per gram cell wet weight (kU $gCWW^{-1}$). 1 mg of RI corresponds to 100 kU [28].

The N-terminal amino acid sequence of processed RI was determined by the Edman degradation procedure in Biocentrum Ltd. (Krakov, Poland) from insoluble protein fraction sample after RI production with DTT in the medium and separation on an 8% SDS-PAGE gel.

The amount of oxidized/reduced DTT in the cultivation medium was determined with the Measure-iT™ Thiol Assay kit (Invitrogen), by following the producer's recommendations.

Acknowledgements

The work was performed in relation with the UNICAT Center of Excellence at the TU Berlin. The research project was financially supported by a research grant of Fermentas UAB. The authors kindly thank Dr. Uwe Horn and Dr. Mario Kraft from Hans-Knöll-Institut Jena, Germany for the periplasmic expression library and the plt1 vector.

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Authors' contributions

JS designed the experimental setup, performed all cultivation experiments and prepared the manuscript. PN initiated the project, assisted with data analysis and manuscript preparation. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 15 February 2011 Accepted: 10 May 2011
Published: 10 May 2011

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doi:10.1186/1475-2859-10-31
Cite this article as: Šiurkus and Neubauer: Reducing conditions are the key for efficient production of active ribonuclease inhibitor in *Escherichia coli*. *Microbial Cell Factories* 2011 **10**:31.

PAPER III

MICROBIAL CELL FACTORIES

RESEARCH Open Access

Heterologous production of active ribonuclease inhibitor in *Escherichia coli* by redox state control and chaperonin coexpression

Juozas Šiurkus[1] and Peter Neubauer[2*]

Abstract

Background: Eukaryotic Ribonuclease inhibitor (RI), belonging to the RNH1 family, is distinguished by unique features - a high sensitivity to oxidation due to the large number of reduced cysteins and a high hydrophobicity, which made most production approaches so far unsuccessful or resulted in very low yields. In this work efficient *in vivo* folding of native RI in the *Escherichia coli* cytoplasm was obtained by external addition of a reducing agent in tandem with oxygen limitation and overproduction of a molecular chaperonin. After optimisation of the production conditions in the shake flask scale the process was scaled up to high cell densities by applying a glucose limited fed-batch procedure.

Results: RI production in a T7 RNA polymerase based system results in accumulation of aggregated inactive product in inclusion bodies. Combination of addition of the reductant DTT, low production temperature and coexpression of the chaperonin GroELS resulted in high level production of approximately 25 mg g^{-1} CDW active RI in *E. coli* ER2566 pET21b, corresponding to approximately 800 kU g^{-1} cell wet weight. Further conditional screening under fed-batch-like conditions with the EnBase® technology and scale up into the bioreactor scale resulted in an efficient high cell density glucose and oxygen limited fed-batch process with a final cell dry weight of 25 g L^{-1} and a total RI yield of app. 625 mg L^{-1} (volumetric activity of 80,000 kU L^{-1}). The *E. coli* based production constructs showed a very high robustness. The recombinant culture maintained its productivity despite the combination of the toxic growth conditions, the substrate limited production mode in tandem with a high level expression of several recombinant proteins, the set of molecular chaperonins and the target protein (RI).

Conclusions: High level production of active RI in *E. coli* in a T7 RNA polymerase expression system depends on the following factors: (i) addition of a reducing agent, (ii) low production temperature, (iii) oxygen limitation, and (iii) co-overexpression of the chaperonin GroELS. The study indicates the strength of applying fed-batch cultivation techniques for the efficient optimisation of production factors already at the screening stage for fast and straight forward bioprocess development even for target proteins which show a complex folding behaviour. In our case none of the approaches alone would have resulted in significant accumulation of active RI.

Background

E. coli is a favorable host for recombinant heterologous protein production. The robustness of this microorganism, fast and simple cultivation, easy genetical manipulation, the enormous amounts of available physiological data and molecular biology tools are key reasons for its widespread use. Despite these positive general characteristics, protein aggregation and/or improper folding are major obstacles which often lead to reduced functional product yields. Many examples indicate the limited capacity of the natural *E. coli* protein folding machinery for a high level accumulation of heterlologous proteins with features wich are not usual for the hosts protein portfolio. Examples are proteins with multiple disulfide bonds, very large proteins, proteins with a high hydrophobicity, and proteins with natural glycosylation. However, target specific engineering approaches and process optimisation can lead to success. In the

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meanwhile there exists an extensive toolbox of elements which overcome natural limitations of the *E. coli* system, such as vectors for coexpression of rare tRNAs, chaperons and foldases, hosts and vectors with improved disulfide oxidation and isomerisation characteristics, newly designed secretion tools, and hosts for superior expression of membrane proteins [1-3]. Even tools for modulation the glycoylation pattern of proteins seems to become a future option [4,5].

Due to their therapeutic importance the production of disulfide stabilised proteins has been a major research focus in the past. This research gave the general impression that the cytoplasm of *E. coli* is a reducing compartment and thus the production of proteins with reduced cysteines should be granted. Hyperoxia and oxydation stress are important factors in connection with protein folding in Eukaryotes (e.g. see recent review by [6]), but have been largely neglected for recombinant protein production in *E. coli*. Although oxidative damage of proteins by carbonylation in *E. coli* has been extensively studied, it has been barely considered in connection with heterologous protein expression. One milestone publication in this aspect is the effect of the dissolved oxygen level on the carbonylation level and activity of human interferon γ [7].

In the present study we show at the example of a protein of the ribonuclease inhibitor (RI) family, that the redox conditions in the cytoplasm are an important target for process optimisation if the aim is the production of soluble and active product.

Proteic RIs are a family of highly conserved proteins. The conservation of the amino acid sequence between different hosts, such as pig, cow, rat, mouse, sheep and human is as high as nearly 70% [8]. All eukaryotic ribonuclease inhibitors share the following characteristics: (i) a high content of reduced cysteins (30-32 residues, 7% of total amino acids), and (ii) a core composed of 15-16 repeated hydrophobic leucine rich motives [8,9]. Due to these features recombinant production of eukaryotic ribonuclease inhibitors has been a challenge. Earlier production trials of human and porcine RI in *E. coli* resulted in low functional yields and major aggregation [10,11]. For example the yield of functional porcine RI in *E. coli* was as low as 10 mg L^{-1} [10] and in *Sacharomyces cerevisiae* 0.2 mg g^{-1} wet weight [12]. Recently we reported production of soluble and functional RI as a fusion to maltose binding protein (MBP) in *E. coli* [13] with a relatively high yield of 39 mg g^{-1} cell dry weight which could be realised in a medium-density fed-batch process with a final yield of app. 800 mg L^{-1} of MBP-RI fusion protein, and correspondingly 425 mg L^{-1} of RI. Similar amounts of functional RI per cell unit from N-terminal fusion proteins were recently obtained by Guo et al. [14], however the authors did not try to maximize the volumetric yield.

Fusion proteins have the disadvantage that an extra proteolytic processing step is needed to obtain the authentic RI, which would be a limitation for industrial scale production. Also, although the tagged protein was soluble, the ~40 kDa MBP fusion partner had a negative impact on the RI specific activity. Therefore in a next study we intended to directly produce almost authentic RI. Therefore we applied two strategies: (i) a cytoplasmic construct with an N-terminal His tag and (ii) a periplasmic construct with a secretion signal which would be removed during the transport of RI into the periplasm [15]. Surprisingly, we only succeeded in high yields of soluble and active RI with the cytoplasmic construct, and only if the reductant dithiothreitol (DTT) was added to the cultivation medium. The same production approach also improved the periplasmic yield of RI. However unexpectedly, the yield of active and soluble RI was higher with the cytoplasmic compared to the periplasmic expression constructs. After further comprehensive optimisation, including different vector constructs and expression principles, the best yield of 320 mg L^{-1} of active His_6-RI was obtained, by combination of the addition of DTT with a low cultivation temperature (22° C) and reduced aeration (pO_2 close to zero) [15].

In the present study we aimed to further increase the cytoplasmic yield of native RI, i.e. without any tag, by applying the very strong routinely used T7 RNA polymerase controlled expression system. Surprisingly, the tested ER2566 (*E. coli* B-strain) pET21b constructs behaved totally different compared to the earlier tested constructs (*E. coli* K-12, plasmids with a *lac*-derived promoter). DTT did not provide a positive effect on the yield of functional RI, possibly due to the imbalance between very strong protein synthesis and slow folding. The problem was solved by co-overexpression of the GroELS chaperonine. The yield of RI finally could be maximized by combining the sequential induction of GroELS and RI with a delayed addition of DTT and the maintenance or a low level of dissolved oxygen. This process strategy also was successful in a bioreactor under fed-batch process conditions with a final cell dry weight of 25 g L^{-1} and a volumetric yield of 625 mg L^{-1} of soluble and highly active RI.

Results

In the present study we were interested to further develop the RI process for cytoplasmic production. Therefore we selected the widely used strong T7 RNA polymerase based expression system based on the vector pET21b and the *E. coli* B strain ER2566 as a production host. In the first set of production trails we applied supplementation with DTT and low production

temperature, as those have been key parameters for active RI production in our previous approach [15]. Surprisingly, contrary to our expectations, DTT did not positively affect soluble accumulation, nor resulted in any higher RI activity in shake flask cultures of *E. coli* ER2566 pET21bRI, independent from the postinduction temperature and DTT addition time (Figures 1 and 2, also see SDS-Page gels in Additional file 1). Despite, a large amount of RI accumulated in inclusion bodies (Additional file 1).

A major difference between the previous and the actual expression systems is the strength of transcription. Previously we used a *lac* derived promoter, but here we used the T7 RNA polymerase based promoter system. The new system supports a very high rate of product synthesis corresponding to more than 60 mg $gCDW^{-1}$ (Figure 2 and Additional file 1) even at the low production temperature. Thus we assumed that the aggregation of RI could be due to the overloading of the cellular folding machinery by the hydrophobic and slowly folding RI. To test this hypothesis we coexpressed the chaperon systems DnaKJE and GroELS in tandem with RI.

While co-overexpression of DnaKJE did not provide any significant improvement in our case, coexpression of GroELS resulted in an increased RI solubility (Figure 2, Additional files 2 and 3) and activity (Figure 1). This positive effect was stronger at a lower postinduction temperature, but the best yield was obtained when DTT was added. The combination of GroELS and DTT resulted in 30-55% improvement of the accumulation of soluble RI (Figure 2 and Additional file 3) and a 1.5 to 2 fold increase in RI activity (Figure 1) compared to the trials without DTT. The best result was obtained when DTT was added two hours after RI induction in combination with a lower postinduction temperature of 30 or 22°C (Figures 1 and 2).

Remarkably, although the highest volumetric activity was obtained with the delayed addition of DTT, the highest activity per cell was obtained when DTT was added at the time of RI induction. In comparison to the processes without DTT the RI activity per cell increased

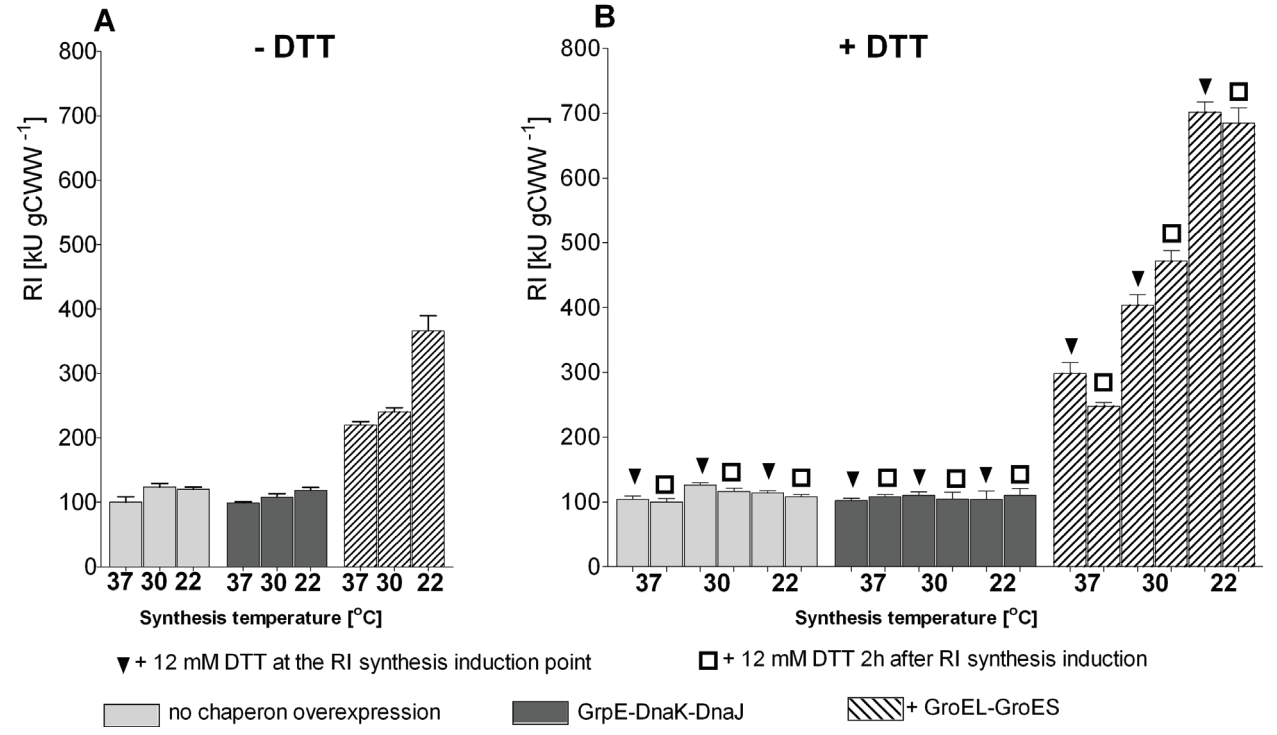

Figure 1 RI protein activities in kilo units per gram of wet cell weight [kU $(gCWW)^{-1}$] after shake flask batch cultivation of *E. coli* ER2566 pET21bRI or with additional chaperone co-overexpression using the pKJE7 (DnaK/DnaJ/GrpE) or pGro7 (GroELS) vectors at 22, 30 or 37°C in glucose-MSM without DTT (A) or with addition of DTT (B). Shake flask cultures were performed in glucose MSM at 37°C until an OD_{600} of 1 to 1.5. Chaperons were induced with 0.4 g L^{-1} of arabinose 2 hours before RI induction (0.2 mM IPTG) at 37°C. The temperature was set at the time of RI induction to 22, 30 or 37°C, respectively. DTT (12 mM final concentration) was added at the time of RI induction (triangles) or 2 hours later (squares). Samples were collected 4 hours after RI induction. Standard deviations represent 2 independent cultivations and 3 assays.

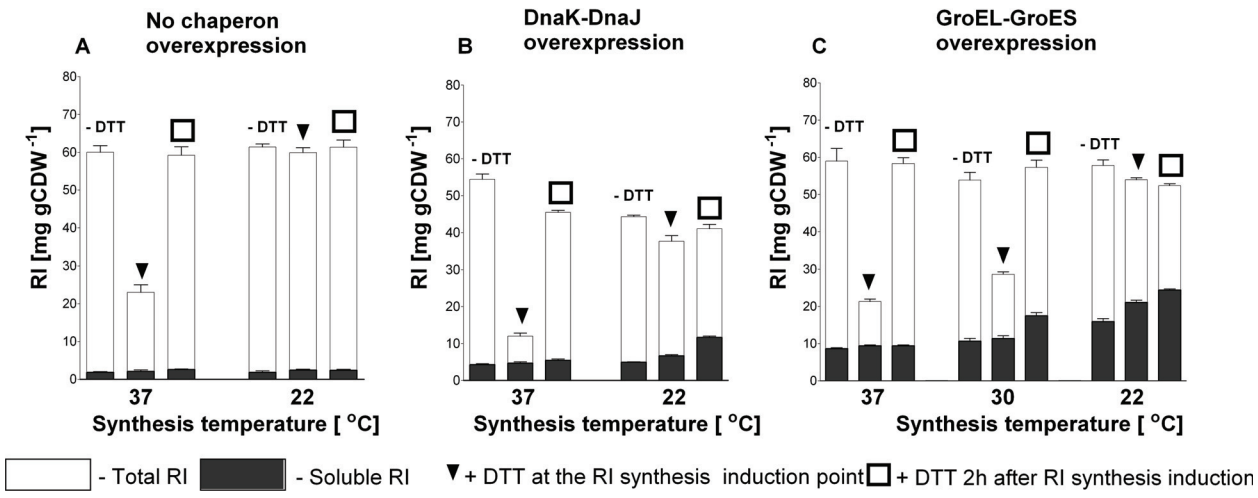

Figure 2 Total (white bars) and soluble (black bars) RI amounts per cell unit [mg (g CDW)$^{-1}$] after 4 hours of batch production in shake flask batch cultures of *E. coli* ER2566 pET21bRI (A) or with additional chaperone co-overexpression using the pKJE7 (DnaK/DnaJ/GrpE) (B) or *pGro7* (GroELS) *(C) vectors* at 22, 30 or 37°C. Cultures were performed in glucose-MSM medium with addition of 12 mM DTT at the time of RI induction (squares), 2 hours after RI induction (triangles), or no addition of DTT (control). Induction of GroELS was performed with 0.4 g L^{-1} of arabinose 2 hours before RI induction (with 0.2 mM IPTG). All cultures were initially started at 37°C and shifted to the respective temperature at the time of RI induction. Standard deviations represent 3 assays.

by 90% when DTT was added at the time of RI induction, but only by 70% in the case of delayed DTT addition (Figure 1).

Interestingly, the combination of DTT and DnaKJE overproduction did only increase the solubility of RI, but the product was principally inactive (Figures 1, 2 and Additional file 2).

In conclusion, (i) the highest activity of RI per cell unit was obtained with a postinduction temperature of 22°C co-expression of GroELS and addition of DTT. (ii)

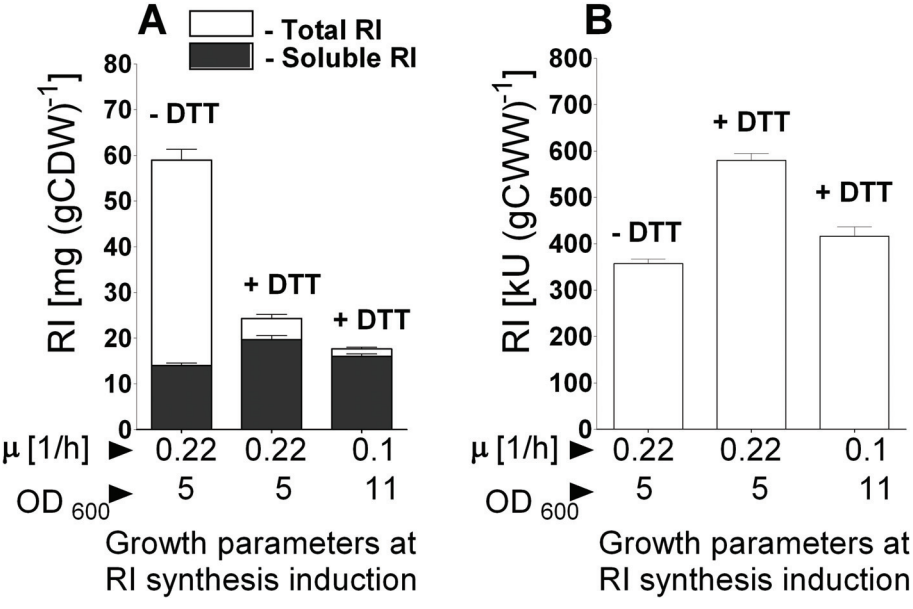

Figure 3 RI protein yields after shake flask EnBase fed-batch cultivation of *E. coli* ER2566 pET21bRI *pGro7* without DTT (-DTT) or with addition of 12 mM of DTT 2 hours after induction of RI (+DTT). Total (white bars) and soluble (black bars) RI amounts in milligrams per gram of dry cell weight [mg (g CDW)$^{-1}$] (A) and RI activities in normalised crude extracts in kilo units per gram of wet cell weight [kU (g CWW)$^{-1}$] (B) 4 hours after RI induction. The amounts of target protein in (A) were determined from scanned SDS-PAGE gel images as described in material and Methods. Standard deviations represent RI amounts from 3 assays.

The delayed DTT addition was preferred in the next set of experiments, as it seemed to be less detrimental to the culture viability.

Fed-batch process in shake flasks

As previously, prior to the bioreactor experiments the fed-batch procedure was basically tested and optimised in shake flasks by applying the EnBase cultivation technique [16]. RI synthesis was induced 2 hours after induction of GroELS by addition of 0.2 mM IPTG, at the fed-batch cultivation mode at $OD_{600} \approx 5$ ($\mu \approx 0.22$ h^{-1}) or $OD_{600} \approx 11$ ($\mu \approx 0.10$ h^{-1}), respectively. Analogical to the batch processes, the cultivation medium was supplemented with DTT at the time of RI induction or 2 hours after induction. RI production was performed at 22°C for 4 hours. The effect of DTT on the production of RI and during co-expression of GroELS was evaluated by comparing the results to a culture without DTT addition.

Interestingly, independent from the cell density at the time of RI induction and in difference to the batch shake flask cultures described above, DTT addition at the time of RI induction had a negative effect on the accumulation of RI, even at the low synthesis temperature (results not shown). In contrast, delayed DTT addition was better, but still the total amount of RI decreased to about 50% in comparison to the fed-batch process without DTT (Figure 3, Additional file 4). However, despite the negative effect of DTT on the total amount of RI per cell (soluble and insoluble fractions), the yield of soluble and active RI per cell was doubled and the final volumetric activity was even five-fold improved compared to the previous batch experiments. Interestingly, the total amount of RI was higher at early induction ($OD_{600} \approx 5$) compared to late induction ($OD_{600} \approx 11$), indicating that the balance of synthesis rate and folding is an important optimisation parameter (Figure 3).

From this part we conclude that the most appropriate fed-batch growth conditions for RI production with GroELS and DTT would be to perform the induction of RI at a specific growth rate μ between 0.1 and 0.22 h^{-1}. Preferably, DTT should be added 1 to 2 hours after RI induction.

Bioreactor process

By taking the previous results into account, the batch and fed-batch RI production processes in the stirred bioreactor with the presence of DTT and GroELS overexpression were designed. Additionally, as a reference culture, also an RI production process without DTT was performed.

RI induction in a glucose limited fed-batch under substrate limitation in the stirred bioreactor cultures was performed at optical densities of app. 20 and 38. It was decided to maintain the specific growth rate in all fed-batch cultures at $\mu \approx 0.22 \pm 0.03$ h^{-1} by an exponential addition rate of the glucose feed solution. As a control also a batch culture was performed with induction of RI at an OD_{600} of 5 ($\mu \approx 0.5$ h^{-1}). In all processes GroELS overexpression was induced 2 hours before induction of the target protein at 37°C (Figure 4) and the temperature was decreased to 22°C at the time of RI induction. Interestingly, in difference to our previously published fed-batch process with the lac-derived promoter system and the *E. coli* K-12 strain [15], in the current cultivations with coexpression of GroELS, the growth of the cultures was not completely inhibited by the addition of DTT, but the cells grew still with $\mu \approx 0.15$ h^{-1} until the end (Figure 4).

By taking the previous study into account, the bioreactor cultivation medium was supplemented with DTT at two different modes; (i) a single DTT pulse addition starting 2 hours after RI induction and (ii) repeated DTT pulses, analogical starting 2 hours after RI synthesis induction. In all bioreactor processes, the air flow was reduced at the DTT addition point, from 30 to 3 - 5 L min^{-1} to maintain the dissolved oxygen concentration in the cultivation medium close to 0%. The stirrer speed was kept the same as before induction (Figure 4).

In agreement with the results from the small-scale study, also in the bioreactor fed-batch processes DTT decreased the accumulation of total RI by 30 to 40% compared to the processes without DTT (Figure 5, Additional file 5). However, despite the negative effect of DTT on the total yield of RI per cell, independently from cultivation mode, DTT improved the soluble amount of RI by 30 to 35%. The RI activity in cultures without DTT was very poor, but a 3.2 - 3.9 fold overall improvement of the activity per cell was obtained with repeated DTT addition compared to the processes without DTT. Interestingly, in contrast to the previously described *E. coli* K-12 process also a single DTT addition caused a good improvement of the RI activity with the new construct (2.2-2.8 fold overall improvement, Figure 5). This was surprising, as the DTT oxidation pattern in this process was similar as in the previously described process of *E. coli* RV308 K-12 [15]. After 3 hours of RI production at the micro-aerobic conditions only 40 to 50% of reduced DTT was detected in the bioreactor medium, and 85 to 90% in a shake flasks process, respectively.

Like in the shake flaks the highest amount of RI per cell unit (≈ 60 mg $gCDW^{-1}$) was achieved in the batch bioreactor without DTT (Figure 5, Additional file 5). The RI amount per cell was approximately 15 - 30% lower during fed-batch operations, depending on the

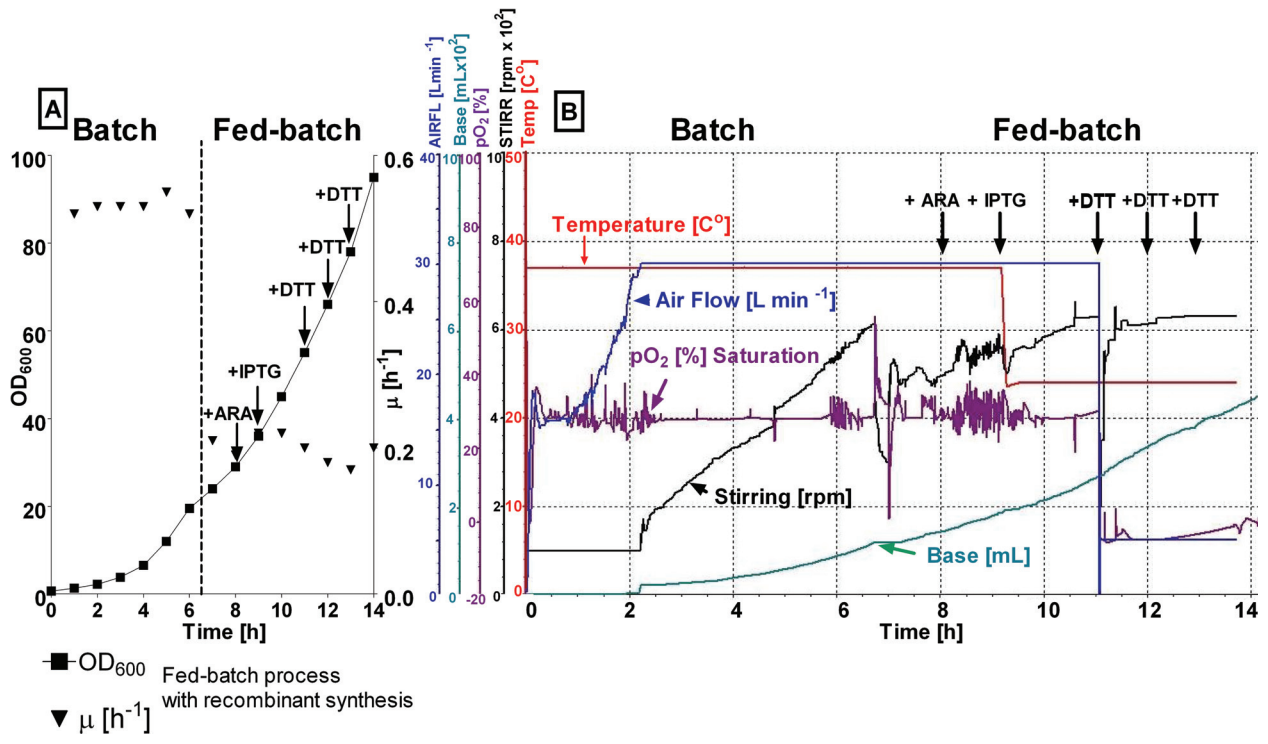

Figure 4 Cell growth (A) and bioreactor parameters (B) of *E. coli* ER2566 pET21bRI *pGro7* during fed-batch RI production with exponential glucose feeding and repeated DTT addition in a 10 L bioreactor.

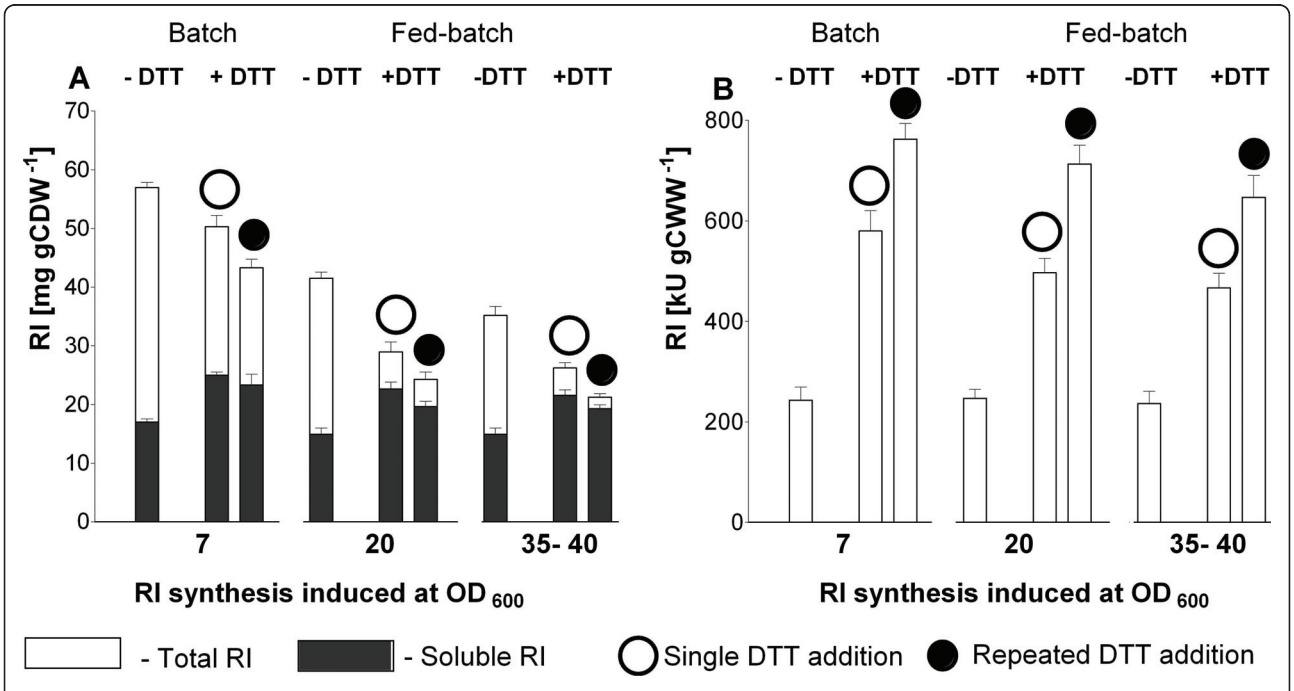

Figure 5 RI amounts and activities normalized per cell amount from samples of batch and fed-batch bioreactor cultivations without and with addition of DTT that was either added once (open circles) or repeatedly (one pulse per hour, filled circles). The first addition of DTT was performed always two hour after induction of RI synthesis. A: Total (white bars) and soluble (black bars) RI amount as mg per g cell dry weight [mg (g CDW)$^{-1}$]. B: RI activities in normalised crude extracts, in kilo units per gram of wet cell weight [kU (g CWW)$^{-1}$]. The data are from 3 independent assays.

cell density at the synthesis induction point (Figure 5, Additional file 5).

Although the RI activity per cell unit was slightly lower in the fed-batch cultures compared to the batch cultures, the volumetric yield of active RI was much higher, especially when RI synthesis was induced at higher OD_{600}. In this case final optical densities of 90 to 95 were reached, corresponding to 23 to 25 g cell dry weight per litre. The fed-batch process with repeated DTT addition yielded 625 mg L^{-1} of RI corresponding to a volumetric activity of ~80,000 kU L^{-1}.

Discussion

In this work we established an efficient recombinant process for Ribonuclease inhibitor (RI) production, resulting in 625 mg L^{-1} of active product with the authentic amino acid sequence. Previously we had improved cytoplasmic and periplasmic folding by either screening for a functioning fusion partner [13] or by controlling the redox situation by external addition of DTT [15] in the *E. coli* RV308 K-12 strain.

Surprisingly, the earlier published approach for cytoplasmic expression of authentic RI only worked in the *E. coli* K-12 system with a P_{lac} promoter, but not in the strong T7 RNA polymerase based expression platform. In the T7 RNA polymerase system all product aggregated, possibly due to the high synthesis rate of RI and thus it outcompetes cellular folding factors. This hypothesis seems to be confirmed by the significant improvement of active RI by co-production of the GroELS chaperonins.

Our attempts to produce RI with chaperons also gave interesting and unexpected results, once more demonstrating the unpredictable effects of chaperon coproduction on the soluble accumulation of target proteins. In our case the most promising DnaKJE chaperone system only resulted in a improvement of RI folding, reflected by increase in the solubility of RI, but the product showed no activity. In contrast, co-production of GroELS had a large positive effect on the soluble accumulation and activity of RI. The best results were achieved at a low production temperature (see Results section Figures 1, 2). Although GroELS co-production has been successfully applied for many recombinant proteins (see recent review by Kolaj et al. [17]), our success was not granted, because there are many reports where GroELS overproduction failed to solubilise or to correctly fold proteins of interest [18-21]. The DnaKJE chaperone system is acting in the early stages of the folding pathway. Thus it possibly stabilizes nascent RI molecules by binding to the hydrophobic patches that stipulates solubility increase, but cannot facilitate complete formation of the native RI horse shoe structure because this would require additional more sophisticated folding. Interestingly, a closer look to the gels shows that DnaK is barely aggregating in tandem with RI, but DnaJ follows RI into the insoluble protein fraction, meaning that DnaJ and RI interact very strongly (Additional file 2). Possibly DnaJ remains bound also in the soluble protein fraction and prevents RI interaction with substrate during the activity assay.

The maximum RI activity and amount in the soluble fraction was achieved in all RI/GroELS co-production experiments at a low post-induction temperature, which fits well with the hypothesis that the cell machinery is easily overloaded by a high production of RI. It may be possible that the increase of solubility and activity of RI, having a size of 49 kD, was obtained by full encapsulation of the target protein within the GroEL barrel, which was stronger expressed in all cases compared to RI (see Figures 3, 4 and 7). Such a shielding RI from the environment may be advantageous to protect it from inactivation by oxidation and may be an explanation for the higher robustness of the GroELS coexpression process for the procedure of when and how DTT is added compared to the previous process where RI was simply expressed in the cytoplasm of *E. coli*.

Interestingly, in all shake flaks batch production experiments, the tandem of RI synthesis induction and DTT addition had a negative effect on the total amount of RI, especially when the production was carried out at higher temperatures. In our opinion, the combination of DTT toxicity, strong RI induction and high production temperature was a strong physiological stress, which drastically diminished cellular resources required for high level RI accumulation. For many other examples such impact of strong induction on cellular growth and recombinant production has been described before. In case of aggregation of the target protein a heat shock like response is induced which leads to accumulation of chaperones and proteases [22-24], changes in cellular respiration [25,26] and even ribosome destruction and loss of viability [27,28]. Interestingly however, somehow the cells in our case were capable to cope with the physiological stress. They maintain RI productivity when the temperature was decreased to 22°C and/or DTT was added only two hours after induction of RI.

Despite its growth inhibiting effect DTT had a positive effect on the solubility and activity of RI during production in shake flasks with GroELS coproduction, possibly by preventing oxidation of cysteins. These data are in good agreement with our previous data of RI production in the *E. coli* K-12 strain, were the dependency of the RI activity on the DTT concentration was demonstrated [15].

Before development of a high cell density bioreactor-scale fed-batch process it was important to test productivity and target protein folding capabilities of the

ER2566 pET21bRI pGro7 construct under substrate limitation. As previously [13], we applied the EnBase fed-batch technique in parallel shake flask trials. We considered that strong co-overexpression of the target protein and the GroELS chaperonin, as well as other factors connected to the recombinant vectors, e.g. expression of the two antibiotic resistance genes, could withdraw critical amount of energy recourses leading to a loss of production capacity under glucose limited cultivation conditions, especially in the presence of toxic DTT. According to this expectation the combination of substrate limitation and DTT decreased the total amount of RI, but the amount of soluble and active RI was even slightly increased. This was also confirmed in bioreactor experiments which were performed by the same principle but at much higher cell densities.

Interestingly, in contrary to the previous study, in EnBase cultures even after a single pulse of DTT, chaperon-mediated RI folding resulted in a twofold higher RI activity compared to the reference process without DTT. However in the bioreactor, despite applying a very low aeration rate, DTT was fast oxidised and thus repeated addition of DTT was absolutely necessary for obtaining a high final product concentration, like in our previous study [15]. However, remarkably the negative effect of DTT oxidation on the folding of RI was not as strong as in the earlier production system, possibly by the shielding effect of GroELS.

In difference to the earlier published process with the K-12 strain and the *lac* promoter system, the cell growth showed a high robustness of the ER2566 pET21-bRI pGro7 clone to the process conditions. After induction cell growth did not cease, but the culture was still growing with a specific growth rate of $\mu \approx 0.15\ h^{-1}$ until the end of the cultivation and was completely consuming the carbon source. This indicates metabolic activity and recombinant productivity at the very high cell densities even after repeated pulses of DTT and a final DTT concentration over 3.5 g L^{-1}. We consider, that co-overexpression of GroELS stipulated such cell robustness against very harsh conditions by saving cellular host proteins which were affected by the high DTT concentrations.

Conclusions

In this work a unique production strategy for RI was established which is based on co-overexpression of GroELS chaperonins, low production temperature and maintenance of reducing conditions. GroELS possibly shields the very slowly folding RI from the environment and thus prevents aggregation of this hydrophobic protein. Also the reducing environment in the GroEL barrel may avoid oxidation of the cysteins of RI. We believe that our strategies may be also important for the folding of other slow folding aggregation-prone proteins. Interestingly, coexpression of GroELS makes the cells more resistant to the toxicity of DTT. This is an interesting aspect that needs further functional investigation.

Methods

Expression strain preparation

The *E. coli* B strain ER2566 (New England Biolabs) was transformed with the plasmid pET21b-RI and was plated on LB agar with ampicillin (100 μg mL^{-1}). The expression strain E. coli ER2566 pET21b-RI was co-transformed with the vectors pGro7 and pKJE7 (Takara Bio Inc) respectively, carrying the genes for the GroEL-GroES and DnaK-DnaJ chaperone systems. Transformants with both plasmids were plated on LB agar containing ampicillin (100 μg mL^{-1}) and chloramphenicol (30 μg mL^{-1}). Both transformations were based on the calcium temperature shock method. Glycerol cell stocks were produced after 8 h of cultivation of the transformants in liquid LB medium with the required antibiotics at 37°C and 220 rpm. A 50% sterile glycerol solution was used to produce 25% glycerol stock cell stocks which were aliquoted in Eppendorf tubes and stored at -70°C.

Cultivation media

Transformations and plasmid propagations were performed on solid and liquid LB medium containing Bacto-Tryptone (10 g L^{-1}), Bacto-yeast extract (5 g L^{-1}), NaCl (10 g L^{-1}), and for solid medium 15 g L^{-1} bacto agar, as well as the required antibiotics. Fed-batch and batch cultivations were performed in glucose-based mineral salt medium (MSM) with the following composition (per litre): Na_2SO_4 2 g, $(NH_4)_2SO_4$ 2.68 g, NH_4Cl 0.5 g, $KHPO_4$ 14.6 g, $NaH_2PO_4 \times H_2O$ 3.6 g, $(NH_4)_2$-H-citrate 1.0 g, and glucose 10 to 15 g. NaOH (40%) was used to adjust pH to 7.0 prior to the heat sterilisation. Additionally, before cultivation the mineral salt medium was supplemented with the following sterile solutions: 3 mL L^{-1} of (1M) $MgSO_4$ and 2 mL L^{-1} of trace element solution with the following composition (per litre): $CaCl_2 \times {}_2H_2O$ 0.5 g, $ZnSO_4 \times 7H_2O$ 0.18 g, $MnSO_4 \times H_2O$ 0.1 g, Na_2-EDTA 20.1 g, $FeCl_3 \times 6H_2O$ 16.7 g, $CuSO_4 \times 5H_2O$ 0.16 g, $CoCl_2 \times 6H_2O$ 0.18 g; as well as 100 μL L^{-1} of thiamine hydrochloride (1 M), 1 mL L^{-1} of ampicilin (100 mg mL^{-1}) and 1 mL L^{-1} of chrolamphenicol (30 mg mL^{-1}). The feeding solution for fed-batch cultivations was based on fully formulated MSM with the required antibiotics and 550 g L^{-1} of glucose.

Batch mode cultivations and recombinant protein synthesis in shake flasks

The inoculums for batch protein production in the shake flasks were prepared by overnight cultivation of

the selected clone in 500 mL shake flaks with 50 ml of MSM medium containing 10 g L^{-1} of glucose at 37°C. For protein production 2 mL of the corresponding inoculum culture was transferred to fresh mineral salt medium containing 10 g L^{-1} of glucose to a final volume of 200 mL in 1L baffled Erlenmeyer shake flasks. Cultures were cultivated at 37°C and 220 rpm until they reached the chaperon over-expression induction point, corresponding to a cell density of OD_{600}≈ 0.5 ± 0.05 (μ = 0.42 ± 0.05 h^{-1}). Induction was performed with of 0.4 g L^{-1} of arabinose. RI was induced 2 hours after chaperon induction with 0.2 mM IPTG. The reducing agent dithiothreitol (DTT) was added to expression cultures as a dry powder to the cultivation medium at the RI induction point or 2 hours after RI induction to achieve a final concentration of 12 mM. The temperature was changed at the RI induction point to 22, 30, or 37°C and the culture was continued for 4 hours at 220 rpm.

Fed-batch mode cultivations and recombinant protein synthesis in shake flasks

The EnBase® technology based fed-batch shake flask cultivations were performed in 1 L baffled Erlenmeyer flasks in 200 mL of MSM as described before by Siurkus et al. [13]. In all experiments the cells were cultivated at the substrate limited mode, generated with 12 AGU L^{-1} of glucoamylase in the medium. GroELS and RI were induced at the two cell densities, which corresponded to the following optical densities and specific growth rates: (1) OD_{600} [GroELS] ≈ 3.0 and μ[GroELS] ≈ 0.22 h^{-1}; OD_{600} [RI] ≈ 5.0 ± 0.2 and μ[RI] ≈ 0.22 h^{-1}; (2) OD_{600} [GroELS] ≈ 9 and μ[GroELS] ≈ 0.15 h^{-1}, OD_{600}[RI] ≈ 11, μ[RI] ≈ 0.1 h^{-1}. With this procedure GroELS synthesis was induced with 0.4 g L^{-1} of arabinose 2 hours before RI. The cultivation temperature and agitation parameters after chaperon induction were maintained at 37°C, 180 rpm. RI induction was performed with 0.2 mM of IPTG, the temperature was shifted to 22°C, and the culture was continued for 4 h at a shaking speed of 180 rpm. DTT was added as a dry powder at the RI induction point or 2 hours after RI induction to a final concentration of 12 mM.

Bioreactor processes

Batch and fed-batch cultures were performed in a 15 L Biostat C bioreactor (B. Braun Biotech, Melsungen, Germany) with an initial cultivation volume of 8 litres. The initial culture parameters as follows: the pO_2 was maintaind at 30% by adapting the stirrer rate and automatic regulation of the air flow (from 0 to 30 liters per min), pH was controlled at 7.0 ± 0.1 by addition of NH_4OH (25%) or H_3PO_4 (2 M). During all bioreactor processes the growth temperature before and after GroELS induction was maintained at 37°C. The temperature in all processes was down-regulated from 37 to 22°C at the RI induction point. A 0.65 M DTT stock solution in MSM was added to the culture 2 hours after RI induction to achieve a final concentration of 12 mM in the cultivation medium. In case of repeated DTT addition a first pulse of 0.65 M DTT stock solution (in MSM) was added to the culture 2 hours after RI induction to achieve final DTT concentration of 12 mM. Additionally two pulses of each 6 mM DTT (final concentration) were added after each of the two following hours.

At the first DTT addition point the air flow was reduced from 30 to 3-4 L min^{-1} and stirring was manually regulated to maintain an oxygen concentration of about 0% in the culture in the presence of DTT (as in [13]). The glucose feeding rate during the fed-batch cultures was controlled by the Biostat software (version 4.62). All processes were monitored by the MFCS/win 2.0 supervisory system. Exponential feeding profiles were programmed to maintain a specific growth rate of μ ≈ 0.22 h^{-1} as earlier described [13].

The fed-batch cultivations were started with a volume of 8.0 L of MSM, and containing 8 or 15 g L^{-1} of glucose, respectively. The fed-batch mode was started after the initial batch cultivation at OD_{600} ≈ 9.5 or OD_{600} ≈ 18, respectively. GroELS were induced 1 hour before RI induction at OD_{600}≈ 12.5-14 or OD_{600}≈ 24-26 in both cases respectively at a specific growth rate μ = 0.22 ± 0.02 h^{-1} under glucose limitation. RI induction during the fed-batch cultures was peformed with 0.2 mM IPTG at OD_{600}≈ 18 or OD_{600}≈ 38 (μ = 0.22 ± 0.02 h^{-1}) and the temperature was shifted to 22°C. The cultures were continued for 5 hours.

The batch cultures were performed in 8 L of MSM medium with 15 g L^{-1} of glucose. The induction of GroELS at the batch cultivation mode was performed at OD_{600}= 3.0 with 0.4 g L^{-1} of arabinose. RI induction with 0.2 mM IPTG was performed one hour later at an OD_{600} of 6.0 (μ ≈ 0.5 h^{-1}) and the temperature was shifted to 22°C. After RI induction the cultures were continued for 5 hours.

Analytical tools

Cell samples harvested from flask and bioreactor cultivations were resuspended in lysis buffer at the following ratio: 1 g of biomass were resuspended in 10 mL of lysis buffer (50 mM Tris-H_3PO_4 pH 8.0, 0.1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 12 mM DTT, 10% propyleneglycol, 0.1 mg mL^{-1} lysozyme). After 30 min of incubation at +4°C the biomass was sonicated for 60 sec (Vibra cell™, Sonic and Materials Inc., sonotrode 6 mm diameter, amplitude 50%) at 4°C. Soluble and insoluble protein fractions were separated by centrifugation for 30 min, 14000 rpm, 4°C. The total protein fraction represents cellular debris suspension (crude extract) before

centrifugation. After centrifugation the insoluble protein pellet was additionally washed and resuspended in the original volume of lysis buffer without lysozyme.

Samples for SDS-PAGE separation were prepared as folllows: 20 μL of protein sample (total soluble, insoluble, protein suspensions), 25 μL of 4 × SDS-PAGE loading buffer (Fermentas), 5 μL of 20 × DTT (Fermentas) and 50 μL of deionized water to obtain a final sample volume of 100 μL. Samples were heated for 15 min at 95°C. 10 μL of sample was applied to each lane of a 10% SDS-PAGE gel.

The amounts in mg of target RI were determined from scanned SDS-PAGE gel images by analysis of the images with TotalLab Quant software (Totallab, Newcaslte, Great Britain). The gels with separated sample proteins were produced for TotalLab quantifications with BSA standards in 3 concentrations on each gel.

The amount of active RI in the soluble protein fraction was determined by an activity assay described by Blackburn et al. [29,30]. One mg of native RI correlates with an activity of about ~100 kU as described by Blackburn et al. [30].

The amount of oxidized/reduced DTT in the cultivation medium was determined by using the Measure-iT™ Thiol Assay kit (Invitrogen) according to the recommendations of the supplier.

Additional material

Additional file 1: SDS-PAGE images of total cell extracts (T), soluble (S), or insoluble (IN) protein fractions normalised to equal cell amounts of *E. coli* ER2566 pET21bRI after 4 hours of batch RI production with addition of 12 mM DTT at the time of RI induction (gels marked with triangles - B, E), 2 hours after RI induction (gels marked with squares - C, F), or no addition of DTT (A, D), respectively. Batch shake flask cultures were performed in glucose MSM at 37 (gel images: A-C), or 22°C (gel images: D-F). Lane abbreviations: 1T - total protein fraction 10 min before induction, 2 (S), 3 (T) and 4 (IN) - soluble, total and insoluble protein fractions 4 hours after RI induction. Protein size marker: PageRuler™™ Protein Ladder Plus (Fermentas).

Additional file 2: SDS-PAGE images of total cell extracts (T), soluble (S), or insoluble (IN) protein fractions normalised to equal cell amounts of *E. coli* ER2566 pET21bRI pKJE7 after 4 hours of batch RI production with addition of 12 mM DTT at the time of RI induction (gels marked with triangles - B, E), 2 hours after RI induction (gels marked with squares - C, F), or no addition of DTT (A, D), respectively. For explanations see Additional file 1.

Additional file 3: SDS-PAGE images of total cell extracts (T), soluble (S), or insoluble (IN) protein fractions of *E. coli* ER2566 pET21bRI pGro7 normalized to equal cell amounts after 4 hours of batch RI production with addition of 12 mM DTT at the time of RI induction (marked with triangles -B, E, H), 2 hours after RI induction (marked with squares-C, F, I), or no addition of DTT (A, D, G), respectively. Batch shake flask cultures were performed in glucose MSM at 37 (gel images: A-C), 30 (gel images: D-F), or 22°C (gel images: G-I). For further explanations see Additional file 1.

Additional file 4: SDS-PAGE images of total cell extracts (T), soluble (S), or insoluble (IN) protein fractions from EnBase fed-batch cultures of *E. coli* ER2566 pET21bRI pGro7 normalized to equal cell amounts after 4 hours of RI production without (A) or with addition of 12 mM DTT (B). RI was induced at OD_{600} of 11. For further explanations see Additional file 1.

Additional file 5: SDS-PAGE images of total cell extracts (T), soluble (S), or insoluble (IN) protein fractions from batch and fed-batch bioreactor cultures of *E. coli* ER2566 pET21bRI pGro7 normalized to equal cell amounts after 4 hours of RI production without (A) or with addition of DTT (B). Gels A and B: protein fractions after RI batch production without (A) and with a single addition of 12 mM DTT (B) added 2 hours after RI induction. Gels C and D: protein fractions after a fed-batch process without (C) and with repeated addition of DTT (D) the first DTT pulse (12 mM) added 2 hours after RI induction. For further explanations see Additional file 1.

Acknowledgements
The work was performed in relation with the UNICAT Center of Excellence at the TU Berlin. The research project was fiancially supported by a research grant of Fermentas UAB belonging to Thermo Fisher Scientific Inc.

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Authors' contributions
JS designed the experimental setup, performed all cultivation experiments and prepared the manuscript. PN initiated the project, assisted with data analysis and manuscript preparation. Both authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 19 March 2011 Accepted: 8 August 2011
Published: 8 August 2011

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doi:10.1186/1475-2859-10-65
Cite this article as: Šiurkus and Neubauer: Heterologous production of active ribonuclease inhibitor in *Escherichia coli* by redox state control and chaperonin coexpression. *Microbial Cell Factories* 2011 **10**:65.

PAPER IV

Proteome profiles for cytoplasmic production of RNase inhibitor in *Escherichia coli* with DTT mediated folding in shake flask and bioreactor cultures

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Introduction

Recently, the folding of oxidation-prone and aggregating eukaryotic ribonuclease inhibitor (RI) in *E. coli* RV308 pCU lacHis_6RI construct was improved by a applying complex expression approach based on redox modification with externally added DTT in combination with low aeration conditions and low synthesis temperature [1]. This study revealed that a single DTT pulse highly improves the accumulation of active RI in shake flasks, but not in the stirred tank-bioreactor, where repeated DTT pulsing for efficient RI folding was required. The possible reason was a higher rate of oxidation of DTT in the bioreactor due to the efficient mixing and aeration, even if the rate of aeration was drastically reduced [1]. However, in a following study, where RI folding was further improved in another strain of *E. coli* by additional coexpression of the chaperones GroEL and GroES even a single DTT pulse in the bioreactor had a positive effect on RI folding [2]. Thus the effect of DTT in combination with a low temperature during the product formation phase and oxygen limitation on the folding RI was not fully clear. A better understanding of the mechanism of the action of DTT in this process, either as a stress inducer or simply as a reducing agent, would be beneficial not only for the production of recombinant RI´s but also would be useful in for the application for other recombinant processes.

The aim of the here presented comparative proteome analysis was to elucidate protein groups which show a significant increase in their expression level, especially redox related proteins when RI is produced in an *E. coli* K-12 strain in these two different systems, i.e. in batch-type shake flasks or in a glucose limited fed-batch process in a stirred-tank bioreactor. This is interesting, as in difference to other expression studies, the optimum recombinant production process for ribonuclease inhibitor is connected to a combination of a number of events which include (i) over-expression of the heterologeous protein (ii) a temperature decrease (ii) cultivation under oxygen limitation, and (iii) medium supplementation with 12 mM of DTT.

This study is interesting, as so far, to our knowledge, no literature data are available which describe the combination of these different stresses. There are several recently published papers describing protein *E. coli* proteome profiles after cell stressing with a single or double stress factors: DTT, 2- hydroxyethyl disulfide (2-HEDS), guanidinium hydrochloride and heat shock [3], benzalkonium

chloride [4], temperature downshift at the high cell densities [5], expression of heterologous glucagon like peptide 1 (GLP-1) [6], and osmotic stress under anaerobic and/or aerobic growth conditions [7]. However, all of these are academic studies and not related to a real production process. As we have recently shown that the conditions applied for production of RNase inhibitor are also relevant for other proteins and as we think, that this procedure might have a wider relevance for recombinant protein production in general, we became interested in understanding the underlying cellular responses in more detail.

Results and Discussion

The proteome analysis of RV308 pCU lacHis$_6$RI constructs

In this work the proteome analysis of the *E. coli* RV308 pCU lacHis$_6$RI construct was carried out in order to investigate protein expression profiles in response to multiple stresses which are induced during RI propagation at optimal and sub-optimal conditions for its soluble accumulation in batch-type shake flasks, and in a substrate limited fed-batch performed in a stirred tank bioreactor, respectively. The changes in the proteome of the *E. coli* RV308 pCU lacHis$_6$RI construct during the cytoplasmic production of RI, temperature downshift, medium supplementation with DTT, and low aeration (in the case of production in the stirred bioreactor) were investigated by using a comparative proteome analysis approach. The 2 DE cytoplasmic protein profiles of non-stressed RV308 pCU lacHis$_6$RI construct, i.e. cells sampled prior the induction of RI, were compared with cell proteomes from the same cultivation processes of samples harvested 3 hours after induction in batch shake flasks of after 5 hours in fed-batch bioreactor experiments, i.e. 3 hours after initiation of low aeration conditions and medium supplementation with DTT (for cultivation and sampling technical details see sections bellow).

The protein spots for MS analysis were selected from the 2 DE gel images by using the Decodon DELTA 2D software, which allowed to elucidate proteins with altered expression levels. In order to ensure consistency of the data and reproducibility of the results the 2DE gels were derived from 3 independed RI production experiments and two 2DE images from each experimental data point. In addition only the protein spots with ≥2 fold change compared to the non-stressed sample (master sample) were considered as consistent and thus were

picked from 2DE gel for tryptic digestion followed by MALDI-TOF-MS analysis. The peptides with a sequence coverage of ≥20% were considered as positive identifications.

The comparative proteome analysis of the stressed culture revealed 57 up-regulated proteins after 3 hours of RI production in the batch-type shake flasks with DTT in the cultivation medium (Figures 1, supplementary figure 1, and table 1). In the fed-batch bioreactor cultures 59 up-regulated proteins were identified after 5 hours of RI synthesis in the glucose limited fed-batch cultivation (Figure 2, supplementary figure 1, table 2). All identified proteins were classified into seven categories according to their physiological roles in the *E. coli K12* cells (see tables 1 and 2). Interestingly, the obtained protein profiles after RI production in the glucose containing batch-type shake flask cultures were partially different from the up-regulated protein profiles obtained in the glucose limited fed-batch cultivations. Only 25 out of over 50 proteins (57 vs. 59) which were strongly induced in both distinct situations were the same.

Response of enzymes of the central carbon and energy metabolism

Pentose phosphate pathway

The comparative proteome analysis showed that RI overexpression under highly reducing conditions in both, the batch-type shake flask cultures and in the glucose limited fed-batch process stipulated a 2-fold increase of the level of transaldolases A and B (TalA/B) belonging to the pentose phosphate pathway which is involved in oxidative stress responses [8].

Interestingly, the increase of transaldolase B in response to recombinant protein production was also reported by several other authors who were using analogical comparative proteome analysis approaches [6, 9]. TalA/B induction could be associated with changes in the cellular redox environment [10] and osmotic stress [7]. Interestingly, a more than 4-fold increase of the decarboxylating 6-phosphogluconate dehydrogenase Gnd, another enzyme of the pentose phosphate pathway enzyme, was found only in the fed-batch cultivation (Figure 2).

Glycolysis

During both, the batch-type shake flask cultures and in the glucose limited fed-batch process the glycolytic enzyme glyceraldehyde-3-phosphate

dehydrogenase GapA was twofold upregulated (Figures 1 and 2). In difference to indicated enzymes of the pentose phosphate pathway, GapA expression is dependent on the alternative heat shock factor σ^{32} [11]. A similar magnitude of GapA up-regulation was found after expression of several other recombinant proteins [6, 9, 12].

AceE and AceF which are components of the pyruvate dehydrogenase multienzyme complex (PDH complex) [13] were found to be approximately 7- and 4-fold up-regulated during RI production in the batch type cultures while only AceF was found to be induced in the glucose limited fed-batch cultures (Figures 1 and 2). The PDH complex catalyses acetyl-CoA synthesis from pyruvate under aerobic conditions, but this complex is rapidly inactivated under anaerobic conditions and oxygen limitation. Thus it is surprising that we find in the actual study an induction of components of this complex. However, also previously in different studies an increase of the level of AceF in *E. coli* BL21 in response to heterologeous protein production was detected during batch production of GLP1 (Lee DH et al. 2007), fed-batch production of leptin and co-expression of the *cysK* gene (Han MJ et. al. 2003). However in difference to our study, these previous reports did not apply oxygen limitation or starvation, respectively.

Probably the low aeration in the bioreactor resulted in a 2-fold up regulation of fermentative D-lactate dehydrogenase (LdhA)(Figure 1), which is known to be induced under oxygen limitation [14, 15].

Finally, RI production in both cultivation systems provoked an increase (2- and 4-fold) of the stress-responsive alternate pyruvate formate lyase subunit YfiD (*grcA*) (Figures 1 and 2). YfiD replaces the oxidatively damaged pyruvate formate lyase subunit [16] and is induced in response to acidic [17] and oxidative stresses [18] under oxygen limitation [19, 20].

A more than 3-fold up-regulation of phosphotransacetylase (Pta) (Figure 1) showed that the cells in the shake flasks are undergoing overflow metabolism due to recombinant expression at very low aeration conditions [21-23]. Interestingly, recently it was described that acetic acid stipulates catabolite repression, which down-regulates acetyl-CoA synthetase and thus by decreasing the acetate assimilation enhances overflow metabolism [24].

TCA cycle enzymes

RI production under reducing conditions and low temperature resulted in a higher content of key TCA cycle enzymes. A drastic increase of 2-oxoglutarate

dehydrogenase (SucA, more than 6-fold) and succinyl-CoA synthethase subunit (SucC, more than 12-fold) were detected during the fed-batch processes (Figure 2). A more than 2-fold increase of the level of succinate dehydrogenase (SdhA) was detected in both processes (Figures 1 and 2) and a 2-fold up-regulation of succinyl-CoA synthetase α subunit (SucD) only in the batch-type shake flask process (Figure 1).

Earlier it was assumed that increased fluxes through the TCA cycle resulted from (i) the metabolic load of the synthesis of a recombinant product and/or (ii) DTT toxicity. Our data are well in agreement with the previously reported proteome analyses which showed that heterologeous protein production induces synthesis of the TCA cycle enzymes SdhA, SucC and SucD in *E. coli* [6, 25-27] and in *Bacillus megaterium* cells [28]. The up-regulation of SucA could be also related with changes in intracellular redox conditions, temperature up-shift [29] and acidic stress [30]. It is worth mentioning that despite the cultivation under oxygen limitation the TCA cycle enzymes were up-regulated at a larger magnitude during the glucose limited fed-batch process in the stirred tank bioreactor compared to the batch-type process in the shake flasks.

Finally, a more than 4-fold up-regulation of citrate synthase (GltA) (Figure 1) was detected. This indirectly confirmed that acetate levels in the cultivation medium of shake flasks increased after induction of the RNase inhibitor compared to the control cultivation (Walsh K et al. 1985). We also believe that the induction of GltA could be related with the induction of the other TCA cycle enzymes. It is worth mentioning that co-expression of citrate synthase allows to decrease acetate accumulation during recombinant protein synthesis [31].

Enzymes of the respiratory chain

Besides induction of the above mentioned proteins belonging to pentose phosphate pathway, glycolysis and TCA cycle enzymes, RI overproduction under glucose excess in the shake flasks resulted in the up-regulation of several proteins belonging to respiratory chain.

The two proteins, belonging to membrane-bound ATP synthase F1 complex – ε subunit (AtpC) and β subunit (AtpD) [32] were up-regulated more than 2-fold compared to the non-stressed culture. Previously, the up-regulation of AtpD was reported in response to denaturing stress elucidated with guanidinium hydrochloride [33]. In addition the increase of the levels of ATP synthase, F1 complex α chain (AtpA) and the β subunit (AtpD) were reported during production

of recombinant human glucagon-like peptide-1 [6], and the ε subunit (AtpC) after overproduction of human leptin [34]. Interestingly, the ATPase delta-subunit (AtpH) was down-regulated in response to overexpression of double-tagged fusion proteins GST-Neu5Ac aldolase-5R and GST-GlcNAc 2-epimerase-5D [25].

Proteins belonging to amino acid and nucleotide biosynthesis and molecular turnover pathways

Cultivation under glucose excess in shake flasks

Besides induction of the above mentioned proteins HisGD, CysK and GshB, RI overproduction under glucose excess in the shake flasks resulted in the up-regulation of another 12 proteins related with amino acid biosynthesis and molecular turnover (Figure 1).

The RI production in the batch-type culture also resulted in a more than 4- and 2-fold up-regulation of class II aminoacyl-tRNA synthetases - alanyl-tRNA synthetase (AlaRS) and Ppolyl-tRNA synthetase (ProRS), respectively. ProRS exhibits natural ability to mischarge $tRNA^{Pro}$ with cysteine resulting in Cys-tRNAPro [35]. It is very likely that the accumulation of this enzyme could be related with the increased demand for tRNAs charged with cysteins due to RI overexpression.

In addition the stressed cells proteome comparison with non-stressed cultures showed that RI production stipulated more than 2-fold up-regulation of 2-dehydro-3-deoxyphosphoheptonate aldolase (AroG), which is involved in the biosynthesis of aromatic amino acids, D-3-phosphoglycerate dehydrogenase (SerA) catalysing the first step in L-serine biosynyhesis, and 2-isopropylmalate synthase (LeuA) catalysing the first reaction of leucine biosynthesis. The LeuA induction could be related with an over-proportional leucine demand due over-expression of the leucine-rich RI.

Besides the above mentioned glutathionylspermidine (GshB) we also found a 3-fold increase of the GSP synthetase encoded by *gss*. An increased level of the GSP synthetase may be related to very low respiration conditions and redox situation in the cell [36, 37].

During the batch and fed-batch cultivations a 4- and 2 fold increase of oxygen-insensitive nitroreductase (NsfA) [38] was detected, respectively (Figures 1 and 3).

In the shaking flasks the 7-α-hydroxysteroid dehydrogenase (HdhA) glutamate-1-semialdehyde aminotransferase (HemL) was upregulated more than 2-fold and 4-fold compared to the non-stressed cultures. The HdhA level in the cell depends on the general stress sigma factor RpoS [39] and is up-regulated in response to osmotic stress [7]. HemL synthesis is regulated by the PhoQ/PhoP system which controls gene expression in response to Mg^{2+} depletion and acidic environment [40], thus the accumulation of this protein could be related with the decreasing pH in the shake flask cultures due to acetate accumulation.

Finally the proteome analysis of the cultures performed in the shake flasks showed a 4-fold up-regulation of uridylate kinase (PyrH) which is involved in pyrimidine biosynthesis [41].

Bioreactor process

In the glucose limited fed-batch process with RI production, medium supplementation with DTT, oxygen limitation and temperature downshift resulted in the accumulation of 16 proteins (Figure 2) assigned to amino acid and molecular turnover pathways.

Argininosuccinate synthetase (ArgG) and Argininosuccinate lyase (ArgH) belonging to the arginine biosynthesis pathway were up-regulated more than 2- and 4-fold, respectively. Previous reports showed that the arginine biosynthesis operon *argCBH* is induced in response to general nutrient stresses and in the stationary phase growth phase [42, 43].

Aspartate aminotransferase (AspC) and Cysteine synthase A (CysK) were up-regulated in the bioreactor process even more than 10- and 5-fold, respectively, compared to the non-stressed cultures (Figure 2). Earlier reports also showed that the AspC is induced in response to guanidinium hydrochloride and temperature up-shift [29]. In addition, it is known that aspartate is directly acting against acidic stress [44] and that it is a precursor for anti heat shock, similar as the osmotic stress agents serine and proline [45]. The increase of the level of CysK could be related with an increased demand for cysteine during the synthesis of RI, as the protein contains 7 % of cysteine. Alternatively, the increase of CysK also may be caused by the stress of the changed redox situation [46].

Despite the very different culture and production conditions under glucose limitation in the bioreactor and glucose excess in the shake flasks, the comparative proteome analysis revealed highly similar up-regulation patterns of

the histidine biosynthesis pathway enzymes histidinal dehydrogenase (HisD) and ATP phosphoribosyltransferase (HisG) [47] by a 2- and 6-fold increase, respectively.

The diguanylate cyclase YddV (DosC) is another drastically (more than 6-fold) up-regulated protein in the glucose limited fed-batch process. The YddV expression is dependent on the general starvation sigma factor σ^s [7]. The two enzymes arabinose 5-phosphate isomerase (KdsD) [48] and DP-N-acetylglucosamine acyltransferase (LpxA) involved in cell wall lipopolysaccharide biosynthesis and lipid A biosynthesis, respectively were up-regulated more than 4 and 2-fold compared to the non-stressed samples (Figure 2). The increased expression level of these cell envelope synthesis enzymes could be related with envelope stress, resulting from RI production. The LpxA and KdsD synthesis levels are related to the envelope stress sigma factor σ^E [49, 50].

The approx. 5-fold up-regulation of the cobalamin-independent homocysteine transmethylase (MetE), an enzyme of the methionine synthesis pathway, in the bioreactor could be related with MetE oxidation by the increasing pool of oxidized DTT in the cultivation medium (Figure 2). Subjected to oxidation MetE is inactivated [51, 52] and up-regulated [53]. Surprisingly our findings show that the sudden oxygen down-shift in the bioreactor did not stipulate MetE synthesis down-regulation which is mediated by anaerobically induced small non-coding RNA known as FnrS [54]. In contrast MetE did not increase in the batch-type shake flask process; here the oxygen level was not specifically down-regulated and also, as no sparging was applied, the oxidation rate of DTT was much lower. It may be worth to mention that acetate in the medium (resulting from overflow metabolism) inhibits the conversion of homocysteine to methionine which is mediated by MetE [55].

The inorganic pyrophosphatase or PPase (Ppa) was up-regulated more than 4-fold in response to RI production in the glucose limited fed-batch process (Figure 2). Previously, a 1.6-fold PPase up-regulation was also reported in response to production of human glucagon-like peptide-1 [6].

Finally, the results showed that RI production in the glucose limited fed-batch process under reducing conditions stipulated a drastic, 11-fold up-regulation of phosphoribosylamine-glycine ligase (PurD) from the purine biosynthesis pathway and a 4-fold increase of homoserine kinase (ThrB) involved in threonine biosynthesis.

Response of proteins related with molecular transport

RI overproduction under reducing conditions in a glucose limited fed-batch process or in a glucose saturated shake flask culture resulted in a higher accumulation of 1 and 7 proteins, respectively, which are involved in molecular transport. In the glucose limited fed-batch process a more than 2-fold increase of oligopeptide ABC transporter subunit OppA was detected. The increase of OppA in response to recombinant protein expression was also reported in previous reports after recombinant protein expression in *E. coli* cells [25] and *Bacillus megaterium* [28]. Interestingly, at the stationary heat shock conditions, the OppA synthesis in *E. coli* was down-regulated [56].

In the shake flask batch process a 2-fold increase of the glucose-specific PTS permease Crr was indicated. This enzyme belongs to phosphoenolpyruvate (PEP)-dependent, sugar transporting phosphotransferase system (PTS). The latest reports showed that Crr is definitely a stress responsive protein. An up-regulation of Crr was previously shown in response to denaturing and redox altering chemicals such as guanidinium hydrochloride, 2-hydroxyethyl disulfide (2HEDS), and DTT [3], at high cell densities (Xiao et al. 2008), and during overexpression of recombinant protein [6, 26]. It is worth mentioning that Crr was utilized as fusion partner for facilitation of recombinant protein folding [57].

The polypeptide Sbp, which is a component of the sulfate ABC transporter CysATWP-Sbp [58] was up-regulated approx. 2-fold in the batch process compared to the non-stressed culture. Interestingly, in a previous study Sbp was transcriptionally up-regulated after hydrogen peroxide treatment [59]. On the proteomic level an increased level of Sbp was found in response to sulfur starvation [60]. One might hypothesize that the high amount of cysteine in RI may be related to sulphur exhaustion and a corresponding upregulation of this protein, however this would need further investigation.

The increase of outer membrane proteins OmpA (approx. 2-fold), OmpC (approx. 6-fold) and OmpX (approx. 4-fold) was detected only during RI synthesis in the shake flasks (Figure 1). The 2-fold up-regulation of OmpA was detected after RI synthesis under glucose-limited fed-batch conditions in the bioreactor (Figure 2). The previous proteomic analysis showed that synthesis of OmpA was increased during the biofilm formation [61]. The up-regulation of OmpA and OmpC was indicated in response to antibiotics [62], for example - nalidixic acid [63] and tetracycline [64] as well as during nitrogen limitation [65]. The previous

proteomic analysis revealed that OmpX is up-regulated in relation to iron availability [66] and changes of external pH (Lauren M et al. 2002). Finally, the recently performed comparative transcriptome and proteome analysis revealed that regulation of expression level of the outer membrane proteins OstA, TolC, OmpT, OmpP OmpC, Trak, Dps, LamB, Tsx, FadL, OmpW, and OmpF are also altered in response to external pH changes [67].

Response of proteins related with heat shock, folding/proteolysis

The RI synthesis under reducing conditions caused a drastic response of δ^{32} depended chaperons, proteases and other stress related proteins. The comparative proteome analysis revealed that the levels of different chaperons, namely the DnaKJ/GrpE chaperon complex (DnaK, GrpE), GroEL/ES chaperonin (GroEL) complexes (Genevaux P et al. 2007), the heat shock protein HtpG from Hsp90 family (Richter K et al. 2007), and the trigger factor (Tig) were increased in response to RI production in all performed cultures (Figures 1 and 2). Interestingly the RI synthesis in the glucose limited fed-batch in the bioreactor process stipulated a drastic, 6-fold up-regulation of DnaK (Figure 1). An earlier proteomic analysis report showed that DTT stipulates up-regulation of DnaK GrpE, GroEL, HtpG, and trigger factor [29]. In addition the same group showed that these proteins are up-regulated in response to 2HEDS (except for HtpG and Tig), GdnHCl, and heat [3].

RI aggregation stipulated the 2- to 4-fold up-regulation of the small molecular weight heat shock proteins IbpA and IbpB during the bioreactor process and an approx. 4-fold increase of IbpB in the shake flasks process, respectively (Figures 1 and 2). IbpA/B are commonly found in inclusion bodies. They are induced by the σ^{32} based heat shock like response and are involved in the protein disaggregation processes of natural aggregates in the turnover of recombinant inclusion bodies[68, 69]. They promote against oxidative stress [70] and protect cellular proteins against thermal inactivation [71].

Furthermore, in both RI production processes highly elevated levels of HslVU protease components were detected: the ATPase HslU and peptidase HslV, and Lon protease as well (Figures 1 and 2). Theses proteases are also controlled by the σ^{32} based heat shock like response [11] and responsible for degradation of misfolded proteins [72, 73]. Also the more than 3-fold increase of ClpB, a

chaperone with proteolytic activity [74] which was observed in both processes, belongs to the σ^{32} based heat shock like response. In assistance with DnaK and the Ibp proteins, ClpB is involved in solubilisation of aggregated proteins [75]. Our data are in good agreement with the results by Gill and co-workers who showed that recombinant protein expression when medium is supplemented with DTT stipulates drastic accumuation of σ^{32}, GroEL, DnaK, ClpB, and also increases activities of cellular proteases [76]. In addition, a recently performed proteome analysis also showed that the σ^{32} based proteases and chaperones ClpB, HslU, GroEL, and IbpA/B were highly induced in response to production of GST tagged GST-GlcNAc 2-epimerase-5D [25].

Besides cytoplasmic chaperons RI production in the fed-batch cultivation also showed an approx. 2-fold higher level of the periplasmic DegP chaperon/protease compared to the time point before RI induction (Figure 2).

Response of proteins related with regulation of cellular redox situation

The 2-fold induction of the periplasmic disulphide oxidase DsbA was only detected in the batch shake flask process (Figure 1). Previously, an approx. 3-fold upregulation of the disulphide bond isomerase DsbD was found in response to 2 HEDS [29]. In addition, after the batch-type shake flask process UvrB, a subunit of the UvrABC nucleotide excision repair complex was 2-fold upregulated (Figure 1). The UvrABC complex belongs to the SOS response proteins and is involved in the repair of damaged DNA [77].

Besides the above mentioned proteins in the shake flasks process we found a 3-fold increase of the GSP synthetase encoded by *gss* and 2-fold increase of glutathionylspermidine (GshB). An increased level of GSP synthetase may be related to very low respiration, i.e. anoxic conditions and reduced redox situation in the cell [36, 37]. The redox related GshB is catalyzing the last reaction in the glutathione synthesis pathway. The inactivation of *gshB* and *trxB* genes leads to growth inhibition. However, the *gshB* disruption effect can be suppressed by medium supplementation with DTT [78].

It is worth to mention that RI production under reducing conditions only in shake flasks resulted in a more than 4-fold accumulation of cystein desulfurase (IscS), an enzyme involved in sulfur metabolism [79].

Also an approx. 2-fold increase of the thiol peroxidase Tpx and of AhpC, a component of the alkyl hydroperoxide reductase, were only detected in the batch-type shake flask process (Figure 1). AhpC is thioredoxin-associated [80] and is acting against stress induced by sulfur limitation [81], xenobiotics [82], and oxidative stress [83]. Previously performed comparative proteome analyses found AhpC to be up-regulated in response to 2HEDS, GndHCl [3], osmotic stress [7, 84], heat [56, 84] and recombinant protein over-expression under batch [6] and fed-batch conditions [85].

The thiol peroxidase Tpx, which performs a mixed-disulfide complex with TrxA, is also an oxidative and stress responsive protein [86, 87]. Previuosly, the Tpx was found to be up-regulated in response to heterologous protein expression [85].

When RI was espressed in the glucose-limited fed-batch bioreactor process a more than 4- and 14-fold accumulation of the SOS and/or oxidative stress responsive proteins UvrY (transcriptional regulator regulator) and DpiB (sensory histidine kinase) were detected (Figure 2). The synthesis increase of inner membrane protein DpiB could be also related with rapidly created low aeration conditions.

RI production in the bioreactor stipulated a more than 5-fold up-regulation of the predicted nitric oxidoreductase YdbC. This enzyme would protect cells from reactive nitrogen species such as nitric oxide [88]. In *E. coli* the regulator NsrR controls the expression of 30 proteins which are acting against reactive nitrogen species [88, 89] including envelope stress sigma factor σ^E depended proteins [90].

The synthesis induction of glutathionine S-transferase encoded by *gstA*, the superoxide dismutases SodA and SodB, and the flavodoxin 1 all were detected in both, the glucose limited fed-batch processes and in the glucose excess batch system (Figures 1 and 2). The RI production under reducing conditions stipulated a more than 2-fold increase of glutathionine S-transferase and a 2- to 7–fold increase of the superoxide dismutases SodA and SodB (manganese and iron superoxide dismutases) (Figures 1 and 2). The up-regulation of these superoxide dismutases is not surprising, because these enzymes act against various stresses, such as oxidative stress [91], heat [56], acidity [92], and they are up-regulated in response to recombinant protein overproduction [6].

Glutathionine S-transferase is known as detoxifying enzyme [93] thus the synthesis increase of this enzyme could be related with DTT toxicity.

Flavodoxin 1 (FldA) was approx. 6-fold upregulated in both processes compared to non-stressed cultures (Figures 1 and 2). FldA is related with the cellular defence against oxidation stress, i.e. FldA synthesis is regulated by the dual transcriptional activator SoxS [94, 95]. In addition, FldA is involved in electron transfer [96] and is essentially for growth under aerobic and anaerobic conditions [97].

Interestingly, the same analysis revealed a more than 2-fold up-regulation of Fur which synthesis is activated by the oxidation stress responsive OxyR and SoxR system [95].

Cold shock responsive proteins

The temperature down-shift in our cultivation systems provoked a drastic increase of proteins involved in transcription/translation initiation and regulation of cold shock responsive genes. The drastic 18- and 8-fold accumulation of the major Cold shock protein CspA was detected in both processes (Figures 1 and 2). Also the ribosome-binding GTPase TypA was up-regulated more than 2-fold in both production processes. TypA is acting on ribosomes structure or function, thus affecting the translation efficiency [98].

The approx. 2-fold accumulation of the chain initiations factors IF2 and IF3 (infB, infC) which are involved in translation initiation of cold shock mRNAs [99] were also detected in both processes (Figures 1 and 2).

The 4- and 6-fold upregulation of the cold responsive proteins CspB and CsdA (*deaD*) was indicated only during the glucose limited fed-batch process (Figure 2). Similar to CspA, CspB is involved in mRNA unfolding at low temperatures for prevention of secondary structure formation [99]. CspB is up-regulated only at much lower temperatures compared to CspA [100]. Therefore we assume that the increase of CspB within the glucose limited fed-batch process was observed due the more precise temperature control, longer incubation at the low temperate conditions and/or faster downshift compared to the simple change of the incubation chamber for the shake flasks. In addition, probably due to the same reasons, the level of CspA was significantly higher in the bioreactor experiments compared to the results obtained in the shake flask cultures (Figures 1 and 2).

Finally, the cold responsive DeaD-box RNA helicase is essential for ribosome biogenesis and mRNA degradation during acclimatisation after temperature

downshift [101]. Interestingly, at the low temperatures the helicase and RNase activities of RNaseR complements with functional activities of CsdA [102].

Response of proteins related with transcription and translation

Besides the mentioned cold shock adaptive proteins also the major translation factor EF-Tu (encoded by *tufB*) was upregulated in both processes (2- to 4-fold) (Figures 1 and 2). Previously, by utilizing comparative proteome analysis, EF-Tu was shown to accumulate in response to heat, DTT, GdnHCl the [3] and overexpression of recombinant proteins [6, 26]. Also, interestingly, an increase of the RNA polymerase subunits α (*rpoA*) and β (*rpoB*) was detected both, in the bioreactor cultivation (Figure 2), and in the shake flask processes (Figure 1), respectively. The accumulation of RpoA and RpoB could be related with stress resulting from RI aggregation. A previous study showed that the RpoA level depends on the presence of the denaturing agent guanidinium hydrochloride [3] and high level production of recombinant proteins, as shown for the 25 kDa protein scFvD1.3 [103].

In addition we found also in the glucose limited fed-batch bioreactor process a number of other proteins to be higher accumulated: the predicted transcriptional regulator YchF (more than 6-fold), the protein chain elongation factor EF-T (Tsf) (more than 5-fold), the transcriptional repressor PurR (more than 4-fold), the 30S ribosomal subunit protein S10 (*rpsJ*) (more than 3-fold), and the transcriptional regulator UvrY (more than 4-fold) (Figure 2).

The previous proteome analyses showed that the protein chain elongation factor EF-T (Tsf) is a stress responsive protein. A significant increase of Tsf was shown in response to the stress agents 2HEDS and guanidinium hydrochloride [3] and high density conditions [3, 104]. It is worth to mention that heterologeous protein folding in *E. coli* cells could be improved by N-terminal fusion to Tsf [29].

Response of other important proteins

After bioreactor process an accumulation of the predicted polypeptide Outer membrane protein YdgH was detected, which also previously was shown to increase in response to recombinant protein expression [6].

The proteomic analysis of the shaking flask process revealed an accumulation of the highly conservative protein TldD. TldD is involved in processing of the

peptide antibiotic Microcin B17, degradation of CcdA which is antidote against toxic CcdB [105], and biosynthesis of pyrroloquinoline quinone (PQQ) [106].

Conclusions

In this work we found that despite the different aeration in the shake flasks and in stirred tank bioreactor the medium supplementation with reducing DTT provoked drastic synthesis increase of oxidative stress responsive proteins, especially in the stirred tank were aeration, although at a low level, was continued after medium supplementation with DTT.

The variety of the detected responses of other proteins was very similar in both processes. However, the induction magnitude of carbon metabolisms and stress the responsive proteins were highly varying depending on the cultivation conditions.

Materials and Methods

Culturing conditions and sampling.

The inoculums for batch RI production in the shake flasks and bioreactor (B. Braun Biotech, Melsungen, Germany) were prepared by overnight batch cultivation of RV308 pCU lacHis_6RI construct [1] in 50 mL (500 mL shaking flasks) and in 500 mL of glucose MSM medium [107] (2 L shake flask) respectively. 5 % of the inoculum culture was transferred to fresh glucose MSM medium (see paper I) to a final volume of 500 mL in 2 L baffled Erlenmeyer shake flasks or to the 10 L stirred bioreactor.

For RI production in the shake flasks the recombinant construct was cultivated in glucose MSM medium with 10 g L^{-1} initial glucose as a batch culture at 37 ℃ and 180 rpm until the induction point was reached corresponding to a cell density of OD_{600}= 1 ± 0.05 (μ ≈ 0.35 h^{-1}). Induction was performed by addition of 1 M IPTG to a final concentration of 0.2 mM. DTT was added to the cultivation medium at the time of RI induction as dry powder to achieve final concentration of 12 mM. Cytoplasmic expression was carried out for 3 h at 22 ℃ at the shaking rate of 180 rpm. The cell samples for 2DE electrophoresis runs were taken 10 min before RI induction (Master Sample) and 3 hours after.

The RI batch production in the stirred bioreactor was carried as described in [1]. The RI synthesis induction was performed at glucose limited mode generated by exponential feeding at OD_{600} of 7± 0.5 (μ ≈0. 22 h^{-1}) as described by Siurkus et al. [1]. RI synthesis was carried for 5 hours at 22 ℃. The cultivation medium

was supplemented with DTT concentrate once, i.e. 2 hours after induction to achieve a final concentration of 12 mM in the cultivation medium. The synthesis was continued for 3 hours under the exponential feeding mode and low aeration conditions which were created by down-regulation of air flow from 30 % pO_2 for maintenance of pO_2 close to 0 % just prior medium supplementation with the DTT as described in Siurkus et al. [1]. The cell samples for 2DE electrophoresis runs were taken 10 min before RI induction ("Master Sample", -10 min) and 3 hours after supplementation of the culture with DTT (in total 5 hours after RI induction).

Preparation of soluble protein extracts, 2 DE protein fractionation and data evaluation

The cells were harvested by centrifugation (22 ℃, 5 min, 10,000 rpm). After centrifugation the cell pellets were immediately frozen in liquid nitrogen. For soluble protein extraction the cell pellets were resuspended in 200 µL of ice-cold cell disruption buffer (50 mM Tris-HCl pH 8.0, 0.1 % Triton X-100, 1 mM EDTA, 1 mM PMSF). The cells were disrupted by sonication for 60 sec with a Vibra cell™ sonicator (Sonic and Materials Inc., 2 mm diameter probe tip) at 4 ℃. Isoelectric focusation (IEF) was performed by using commercial IPG strips (GE Healthcare) with a pH range of 3 to 10. 200 µg of soluble cellular proteins were loaded per IPG strip and separated according to izoelectric point as described previously [108]. The second dimension of separation, according protein mass, was performed as described by Voigt et al. [109]. The gels were stained with Flamingo Fluorescent gel stain (Biorad) by following the manufacturer´s recommendations. The 2D gel image comparison with the "master sample" gel for protein spot selection and identified protein labelling on the gel images was performed by using Decodon DELTA2D software. The selected protein spots (by using Decodon DELTA2D software) were cut with a Spot Cutter system (Biorad). Prior to MALDI-TOF MS, analysis the selected proteins were digested in the gel with trypsin. The MS data were treated with "Peak-to-Mascot" script of 4700 Explorer ^{TM} software (Applied Biosystems) and protein identification from the Mascot database was performed by using the Mascot search engine (Matrix Science).

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Figure Legends

Figure 1. The up-regulation levels of soluble *E. coli* K12 RV308 pCUlac His_6RI construct proteins after 3 hours of RI synthesis in the shake flasks under highly reducing conditions at 22 ℃. The "-10" and "+180" are representing harvested samples 10 min before and 180 min after RI induction, temperature downshift, and addition of DTT. The upregulation levels of the identified proteins were estimated from overlaid 2DE images by using the Decodon DELTA2D software. The data are derived from 3 independent cultivation experiments. The up-regulated proteins are subdivided into 7 categories according their physiological function in *E. coli* MG1655 K-12.

Figure 2. The up-regulation levels of soluble *E. coli* K12 RV308 pCUlac His_6RI construct proteins after 5 hours of RI synthesis in the stirred bioreactor at 22 ℃ under highly reducing conditions and low-aeration. The "-10" and "+300" are representing harvested samples 10 min before RI induction and 300 min after induction, i.e. 180 min after initiation of low aeration conditions and addition of DTT. The aeration was down-regulated from a pO_2 of 30 % to maintain 0 % in the cultivation medium. The temperature downshift from 37 to 22 ℃ was performed at the RI induction point. For further explanation see legend of Fig. 1.

Supplementary figure 1. Overlaid 2DE images derived by using DELTA2D software of soluble E. coli K12 RV308 pCUlacHis_6RI proteomes before [-10 min] and after [+180 min] 3 hours of RI induction in the shake flasks. RI synthesis was performed at the 22 ℃, the reducing conditions were created by addition of 12 mM DTT to the cultivation medium at the RI induction point.

Supplementary figure 2. Overlaid 2DE images derived by using DELTA2D software of soluble E. coli K12 RV308 pCUlacHis_6RI proteomes before [-10 min] and after [+300 min] 5 hours of RI induction, i.e. 180 min after medium supplementation with DTT in stirred tank bioreactor. RI synthesis was performed at 22 ℃. The air flow was down-regulated and the reducing agent was added at the same time point, i.e. 2 hours after RI induction.

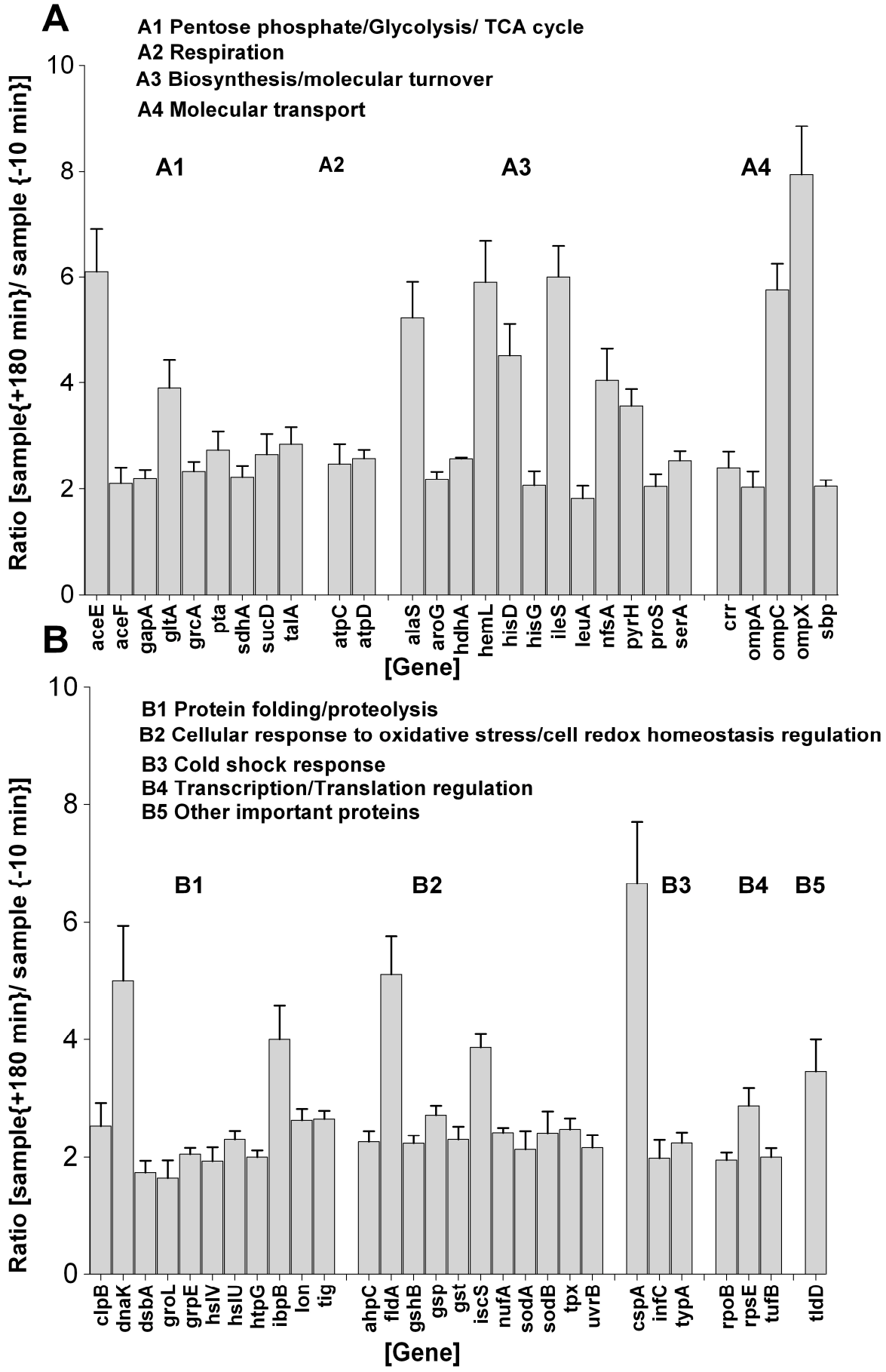
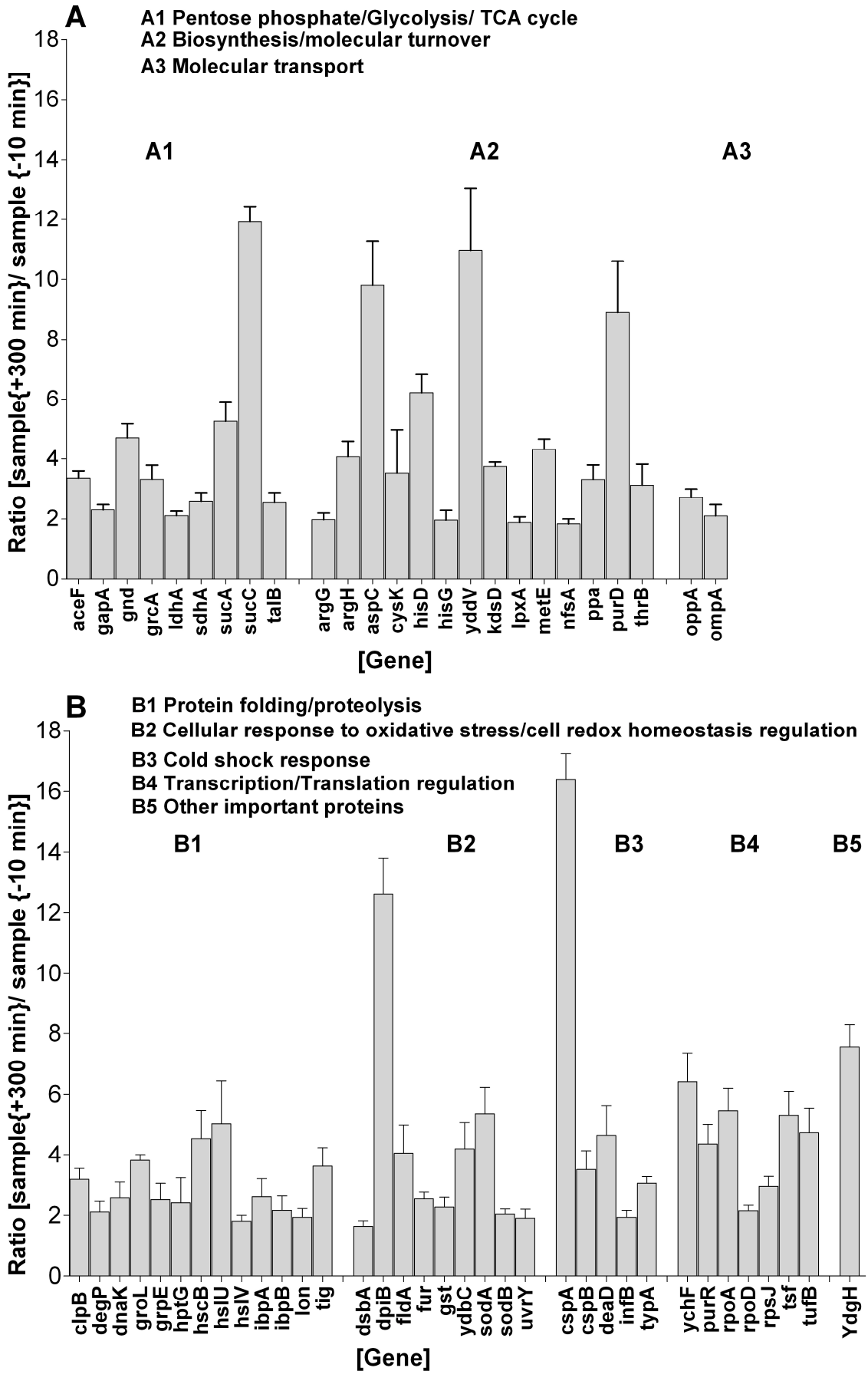

A
A1 Pentose phosphate/Glycolysis/ TCA cycle
A2 Respiration
A3 Biosynthesis/molecular turnover
A4 Molecular transport
A1
A2
A3
A4
Ratio [sample{+180 min}/ sample {-10 min}]
[Gene]
aceE aceF gapA gltA grcA pta sdhA sucD talA atpC atpD alaS aroG hdhA hemL hisD hisG ileS leuA nfsA pyrH proS serA crr ompA ompC ompX sbp
B
B1 Protein folding/proteolysis
B2 Cellular response to oxidative stress/cell redox homeostasis regulation
B3 Cold shock response
B4 Transcription/Translation regulation
B5 Other important proteins
B1
B2
B3
B4
B5
Ratio [sample{+180 min}/ sample {-10 min}]
[Gene]
clpB dnaK dsbA groL grpE hslV hslU htpG ibpB lon tig ahpC fldA gshB gsp gst iscS nufA sodA sodB tpx uvrB cspA infC typA rpoB rpsE tufB tldD

Figure 1


A
A1 Pentose phosphate/Glycolysis/ TCA cycle
A2 Biosynthesis/molecular turnover
A3 Molecular transport
A1
A2
A3
Ratio [sample{+300 min}/ sample {-10 min}]
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2
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12
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aceF
gapA
gnd
grcA
ldhA
sdhA
sucA
sucC
talB
argG
argH
aspC
cysK
hisD
hisG
yddV
kdsD
lpxA
metE
nfsA
ppa
purD
thrB
oppA
ompA
[Gene]
B
B1 Protein folding/proteolysis
B2 Cellular response to oxidative stress/cell redox homeostasis regulation
B3 Cold shock response
B4 Transcription/Translation regulation
B5 Other important proteins
B1
B2
B3
B4
B5
Ratio [sample{+300 min}/ sample {-10 min}]
0
2
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12
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18
clpB
degP
dnaK
groL
grpE
hptG
hscB
hslU
hslV
ibpA
ibpB
lon
tig
dsbA
dpiB
fldA
fur
gst
ydbC
sodA
sodB
uvrY
cspA
cspB
deaD
infB
typA
ychF
purR
rpoA
rpoD
rpsJ
tsf
tufB
YdgH
[Gene]

Figure 2

Table 1. The up-regulated proteins in response to RI synthesis in shaking flasks at the low temperature and under reducing conditions identified through comparative proteome analysis by using MALDI-TOF-MS

Nb	Gene name*	Protein Name [Escherichia coli K12]*	Accession Number*	Theoretical Protein MW [Da]	Theoretical Protein PI	Coverage [%]
Pentose Phosphate/Glycolysis/TCA cycle						
1	aceE	Pyruvate dehydrogenase (decarboxylase component)	P0AFG8	99606	5.46	29
2	aceF	Pyruvate dehydrogenase (dihydrolipoyltransacetylase component)	P06959	66055	5.09	45
3	gapA	Glyceraldehyde-3-phosphate dehydrogenase A	P0A9B2	35510	6.61	61
4	gltA	Citrate synthase	P0ABH7	47984	6.21	54
6	pta	Phosphotransacetylase	P0A9M8	77124	5.28	28
7	sdhA	Succinate dehydrogenase, flavoprotein subunit	P0AC41	64381	5.85	35
8	sucD	Succinyl-CoA synthetase, α subunit	P0AGE9	29759	6.32	55
9	talA	Transaldolase A	P0A867	35636	5.89	55
Respiration						
10	atpC	Membrane-bound ATP synthase, F1 sector, ε-subunit	P0A6E6	15059	5.46	71
11	atpD	Membrane-bound ATP synthase, F1 sector, β subunit	P0ABB4	50294	4.9	67
Biosynthesis, molecular turnover						
12	alaS	Alanyl-tRNA synthetase	P00957	95973	5.53	35
13	aroG	3-deoxy-D-arabinoheptulosonate-7-phosphate synthase	P0AB91	37986	6.14	65
14	hdhA	NAD-dependent 7 α-hydroxysteroid dehydrogenase	P0AET8	26762	5.22	33
15	hemL	Glutamate-1-semialdehyde aminotransferase	P23893	45336	4.73	50
16	hisD	Histidinol-phosphate aminotransferase	P06988	46082	5.06	24
17	hisG	ATP phosphoribosyltransferase	P60757	33345	5.47	59
18	ileS	Isoleucine tRNA synthetase	P60757	104231	5.66	23
19	leuA	3-isopropylmalate isomerase (dehydratase) subunit	Q46857	49850	5.9	61
20	nfsA	NADPH nitroreductase	P17117	26784	6.45	58
21	pyrH	Uridylate kinase	P0A7E9	25954	6.85	58
22	proS	Proline tRNA synthetase	P16659	63653	5.12	47
23	crr	Glucose-specific phosphotransferase enzyme IIA component	P69783	18240	4.73	50
24	ompA	Outer membrane protein A	P0A910	37178	5.99	65
25	ompC	Outer membrane protein C	P06996	40343	4.58	51
26	ompX	Outer membrane protein X	P0A917	18591	6.56	63
27	sbp	Periplasmic sulfate-binding protein	P0AG78	36637	6.62	27

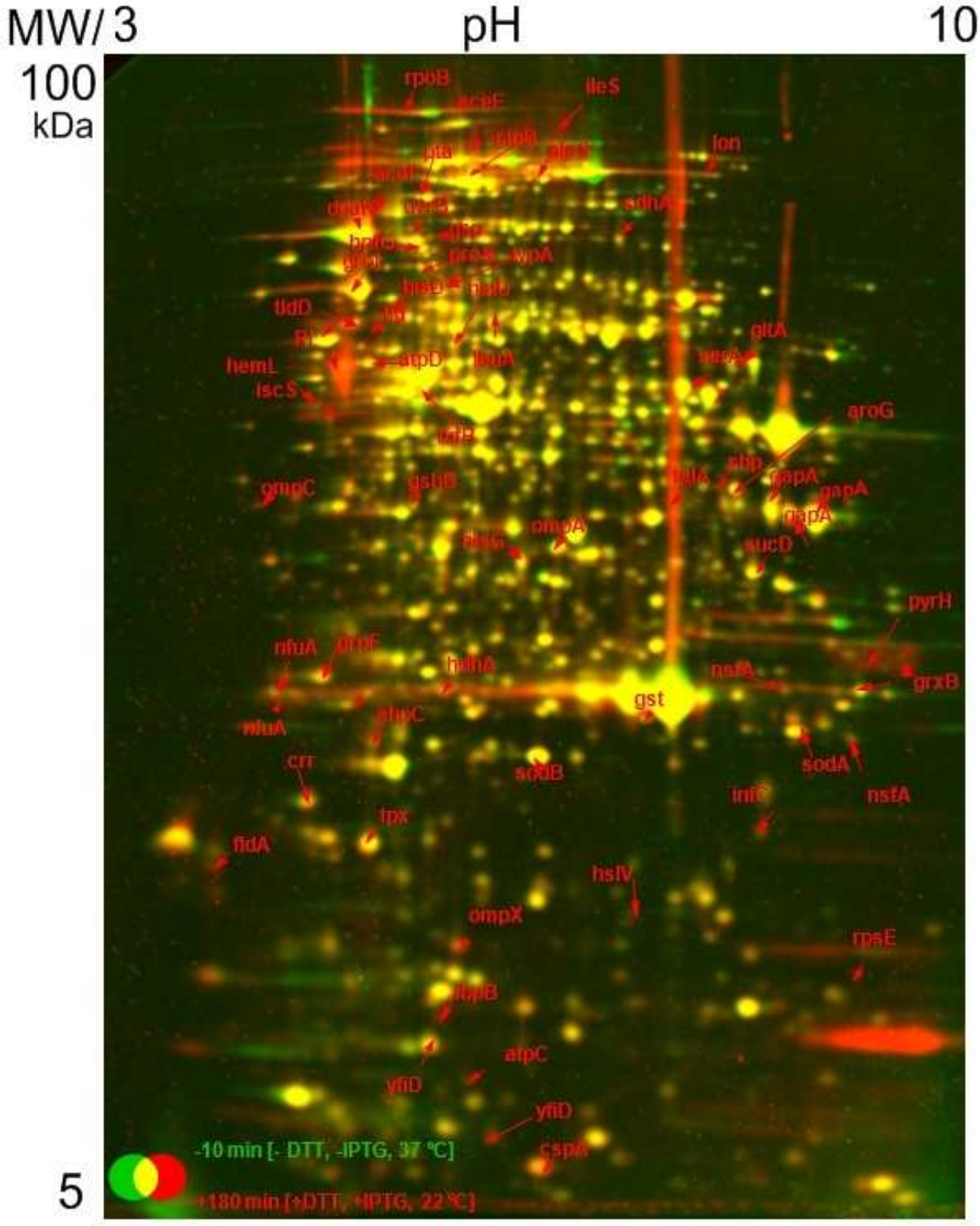
28	clpB	Chaperone protein ClpB	P63284	95526	5.37	28
29	dnaK	Chaperone Hsp70;	P0A6Y8	69072	4.83	56
30	dsbA	Periplasmic protein disulfide isomerase I	P0AEG4	23090	5.95	38
31	yfiD	Putative formate acetyltransferase grcA (YfiD)	P68066	14275	5.09	59
32	groL	60 kDa chaperonine	P0A6F5	57293	4.85	68
33	grpE	Protein grpE	P09372	21784	4.68	37
34	hslV	ATP-dependent protease HslV	P0A7B8	19081	5.96	42
35	hslU	ATP-dependent hsl protease ATP-binding subunit HslU	P0A6H5	49550	5.24	53
36	htpG	Chaperone Hsp90, heat shock protein C 62.5	P0A6Z3	71378	5.09	38
37	ibpB	Small heat shock protein	P0C058	16315	5.19	61
38	lon	ATP-dependent protease La	P0A9M0	87383	6.01	29
39	tig	Trigger factor	P0A850	48163	4.83	53
Cellular response to oxidative stress/cell redox homeostasis regulation						
40	ahpC	alkyl hydroperoxide reductase, C22 subunit	P0AE08	20748	5.03	50
41	fldA	Flavodoxin 1	P61949	19725	4.21	43
42	gshB	Glutathione synthetase	P04425	35538	5.11	67
43	gsp	Glutathionylspermidine synthetase/amidase	P0AES0	70487	5.13	32
44	iscS	Cysteine desulfurase	P60757	46032	6.04	41
45	gst	Glutathionine S-transferase	P0A9D2	22854	5.85	53
46	nufA	Protein: iron-sulfur cluster scaffold protein	P63020	20984	4.52	40
47	sodA	Superoxide dismutase [Mn]	P00448	23083	6.45	62
48	sodB	Superoxide dismutase [Fe]	P0AGD3	21253	5.58	39
49	tpx	Thiol peroxidase	P0A862	17824	4.75	42
50	uvrB	UvrABC system protein B	P0A8F8	76178	5.14	42
Cold shock response						
51	cspA	Cold shock protein cspA	P0A9X9	7399	5.58	77
52	infC	Protein chain initiation factor IF-3	P0A707	20551	9.54	78
53	typA	GTP-binding protein TypA/BipA	P32132	67313	5.16	24
Transcription/translation regulation						
54	rpoB	RNA polymerase, δ(70) factor	P0A8V2	150538	5.15	27
55	rpsE	30S ribosomal subunit protein S5	P0A7W1	17592	10.11	79
56	tufB	Protein chain elongation factor EF-Tu (	P0CE48	43286	5.3	36
Other important proteins						
57	tldD	Protein tldD	P0AGG8	51332	4.93	36

Table 2. The up-regulated proteins in response to RI synthesis in stirred bioreactor at the low temperature, low aeration and under reducing conditions identified through comparative proteome analysis by using MALDI-TOF-MS

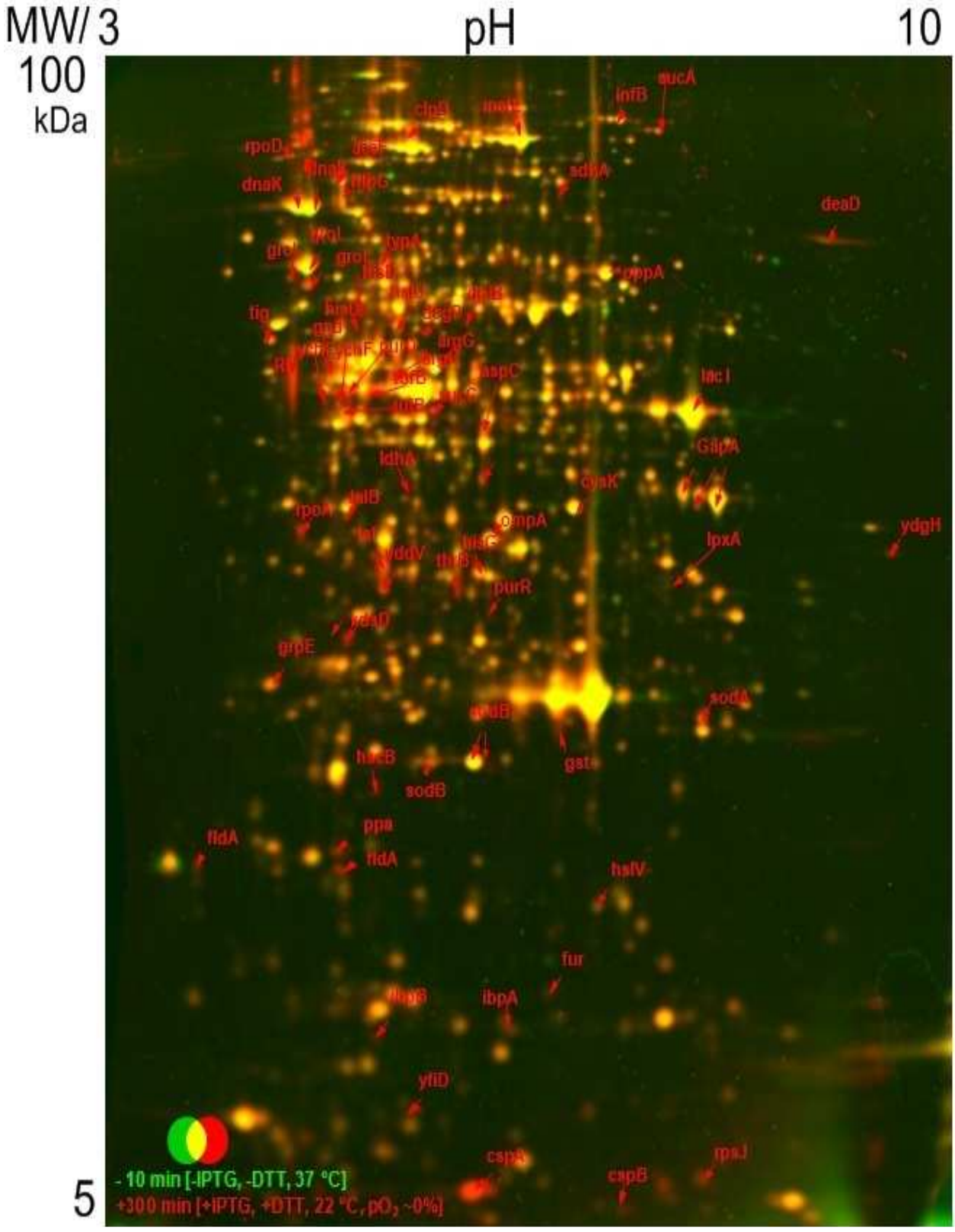
Nb	Gene name*	Protein Name [Escherichia coli K-12]*	Accession Number*	Theoretical Protein MW [Da]	Theoretical Protein PI	Coverage [%]
Pentose Phosphate/Glycolysis/TCA cycle						
1	aceF	Acetyltransferase component of pyruvate dehydrogenase	P06959	66055	5.09	22
2	gapA	Glyceraldehyde-3-phosphate dehydrogenase A	P0A9B2	35510	6.61	38
3	gnd	6-phosphogluconate dehydrogenase, decarboxylating	P00350	51497	5.1	27
4	grcA	Putative formate acetyltransferase (YfiD)	P68066	14275	5.09	60
5	ldhA	Fermentative D-lactate dehydrogenase, NAD-dependent	P52643	36511	5.29	48
6	sdhA	Succinate dehydrogenase, flavoprotein subunit	P0AC41	64381	5.85	25
7	sucA	2-oxoglutarate dehydrogenase	P0AFG3	104996	6.04	18
8	sucC	Succinyl-CoA ligase [ADP-forming] subunit β	P0A836	41367	5.37	22
9	talB	Transaldolase B	P0A870	35197	5.11	26
Biosynthesis, molecular turnover						
10	argG	Argininosuccinate synthetase	P0A6E4	49867	5.23	42
11	argH	Argininosuccinate lyase	P11447	50287	5.11	28
12	aspC	Aspartate aminotransferase	P00509	43546	5.54	67
13	cysK	Cysteine synthase A, O-acetylserine sulfhydrolase A	P0ABK5	34468	5.83	62
14	hisD	L-histidinal:NAD+ oxidoreductase	P06988	46082	5.06	33
15	hisG	ATP phosphoribosyltransferase	P60757	33345	5.47	38
16	yddV	Diguanylate cyclase	P0AA89	53145	5.89	30
17	kdsD	Arabinose 5-phosphate isomerase	P45395	35173	6.22	33
18	lpxA	UDP-N-acetylglucosamine acetyltransferase	P0A722	28062	6.63	37
19	metE	homocysteine methyltransferase	P25665	84621	5.61	21
20	ppa	Inorganic pyrophosphatase	P0A7A9	19691	5.03	31
21	purD	Phosphoribosylamine--glycine ligase	P15640	45911	4.96	36
22	thrB	Homoserine kinase	P00547	33602	5.45	33
23	oppA	Periplasmic oligopeptide-binding protein	P23843	60861	6.05	36
24	ompA	Outer membrane protein A	P0A910	37178	5.99	56
25	clpB	Chaperone protein ClpB	P63284	95526	5.37	23
26	degP	Protease Do	P0C0V0	47176	5.76	45
27	dnaK	Chaperone protein DnaK	P0A6Y8	69072	4.83	28
28	groL	60 kDa chaperonin	P0A6F5	57293	4.85	37
29	grpE	Protein GrpE	P09372	21784	4.68	50

30	htpG	Chaperone protein HtpG	P0A6Z3	71378	5.09	43
31	hscB	Co-chaperone protein HscB	P0A6L9	20125	5.05	52
32	hslU	ATP-dependent hsl protease ATP-binding subunit HslU	P0A6H5	43286	5.3	31
33	hslV	ATP-dependent protease HslV - Escherichia coli	P0A7B8	19081	5.96	45
34	ibpA	Small heat shock protein ibpA	P0C054	15764	5.57	63
35	ibpB	Small heat shock protein ibpB	P0C058	16315	5.19	63
36	lon	ATP-dependent protease La	P0A9M0	87383	6.01	23
37	tig	Trigger factor	P0A850	48163	4.83	48
Cellular response to oxidative stress/cell redox homeostasis regulation						
38	dsbA	Thiol:disulfide interchange protein DsbA	P0AEG4	23090	5.95	34
39	dpiB	Sensor kinase DpiB	P77510	61645	5.77	25
40	fldA	Flavodoxin-1	P61949	20748	5.03	41
41	fur	Ferric uptake regulation protein	P0A9A9	16784	5.68	64
42	gst	Glutathionine S-transferase	P0A9D2	22854	5.85	53
43	ydbC	Predicted oxidoreductase	P25906	30687	5.32	50
44	sodA	Superoxide dismutase [Mn]	P00448	23083	6.45	37
45	sodB	Superoxide dismutase [Fe]	P0AGD3	21253	5.58	42
46	uvrY	Putative 2-component transcriptional regulator	P0AED5	23877	6.53	38
Cold shock response						
47	cspA	Cold shock protein CspA	P0A9Y0	7399	5.58	77
48	cspB	Cold shock-like protein CspB	P36995	7712	6.54	78
49	deaD	Cold-shock DEAD box protein A	P0A9P6	72669	8.71	26
50	infB	Translation initiation factor IF-2	P0A705	97290	5.8	21
51	typA	GTP-binding protein TypA/BipA	P32132	67313	5.16	28
Transcription/translation regulation						
52	ychF	GTP-dependent nucleic acid-binding protein EngD	P0ABU2	39642	4.87	31
53	purR	HTH-type transcriptional repressor PurR	P0ACP7	38150	6.28	26
54	rpoA	DNA-directed RNA polymerase subunit α	P0A7Z4	36489	4.98	48
55	rpoD	RNA polymerase, δ(70) factor	P00579	70219	4.69	33
56	rpsJ	30S ribosomal subunit protein S10	P0A7R5	11728	9.68	38
57	tsf	Elongation factor Ts	P0A6P1	30404	5.22	74
58	tufB	Protein chain elongation factor Tu (duplicate of TufA)	P02990	43286	5.3	61
Other proteins						
59	ydgH	Protein YdgH	P76177	33852	9.27	61

* –the gene name, protein name and accession number is based on the data from UniProtKB protein database http://www.uniprot.org/



Supplementary figure 1



Supplementary figure 2