Cellular responses to the induction of recombinant genes in *Escherichia coli* fed-batch cultures

Dissertation

in fulfillment of the requirements for
the degree of

doctor rerum naturalium (Dr. rer. nat.)

submitted to the

Martin-Luther-Universität Halle-Wittenberg
Faculty of Mathematics and Natural Sciences
Department of Biochemistry and Biotechnology

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February 2000

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Halle (Saale), 28.02.2000

Lin, Hongying (1999). **Cellular responses to the induction of recombinant genes in *Escherichia coli* fed-batch cultures**. Department of Biochemistry and Biotechnology, Martin-Luther-Universität Halle-Wittenberg, Halle (Saale), Germany

Abstract

This thesis concerns the investigation of cell growth, plasmid stability and amplification, recombinant protein overproduction, cellular metabolism, and several stress responses after induction in glucose limited fed-batch recombinant cultures. The extensive study was specifically focused on one process for production of a yeast α-glucosidase in *E. coli* RB791 by derepression of the P_{tac} promoter with IPTG. This investigation was also compared to other model systems, including CRIMI (creatinine imino hydrolase) in a small scale cultivation and ZZ protein (a modified domain B of staphylococcal protein A) in a large-scale process.

Induction of α-glucosidase formation led to an inhibition of cell growth and glucose uptake. The growth inhibition was connected to a decrease of the colony forming ability of the cells, which declined to approximately 5 % within 4 h after induction in the strain with coexpression of argU-tRNA. The non-culturable cells were shown to have not lost all metabolic activities, and even succeed to maintain some glucose uptake and respiratory ability. The ability of these cells for replication is apparently not only impaired by competition of the synthesis of the recombinant product to the formation of cellular house-keeping proteins, but specifically by continued damage of the chromosomal DNA or loss of superhelicity. The cells are unable to induce the SOS response, as the product formation occupies a large part of the protein synthesis machinery, and consequently the cells loose their ability to recover irrevocably. This model of the inability of cells to have not the opportunity to respond to DNA damage is new in view of recombinant protein production.

Furthermore, up-growth of plasmid-free cells was observed in the α-glucosidase process. The maximal glucose uptake capacity decreased to only about 25 % of the q_{smax} of the batch phase. Reduction of q_{smax} may be a serious problem in recombinant fed-batch processes, because it results in overfeeding of substrate which in a turn supports the up-growth of plasmid-free cells and therefore lowers the productivity.

After induction, the recombinant plasmid pKK177glucC was amplified by a factor of three to five. The plasmid copy number increased from about 100 to 300-400 per cell within a period of six hours in glucose-limited fed-batch cultivations. In contrast, no amplification occurred if product formation was not induced. Cultures with the same *E. coli* strain, but other recombinant ampicilline based plasmids, were also overgrown by plasmid-free cells, when the growth was inhibited by overexpression of the recombinant genes, but showed no up-growth of plasmid-free cells and no plasmid amplification when product formation did not inhibit growth.

Glucose limited fed-batch cultivations of *Escherichia coli* cells are characterized by a transient increase of the stringent response regulator ppGpp (guanosine-3',5'-bisphosphate), a higher concentration of the general stress response regulator σ^S and an accumulation of extracellular cAMP during the shift from unlimited to limited growth. The influence of the overexpression of recombinant genes on the concentration of these stress regulators was compared in different expression systems. It has been shown that the response can be different in dependence on the recombinant gene. In case of the α-glucosidase process, no general stress response was induced, and the concentration of the three regulators (ppGpp, σ^S and cAMP) decreased to very low levels. In contrast, induction of the recombinant CRIMI caused a strong increase of σ^S and continuous accumulation of cAMP in the cultivation medium. Although the different products were accumulated to similar levels in these various systems, significant differences were also detected in connection to the influence of the recombinant production on the cellular growth and cell survival. The results suggest that induction strength on the transcriptional level and the strength of the ribosome binding site, but specifically the gene codon usage of a recombinant gene influence the behavior of stress signals.

The small scale process of α-glucosidase was also investigated by a down-scale procedure, where glucose oscillations were created by an on/off feeding mode in either short cycles (1 min) or long cycles (4 min). The influence of repeated short-term glucose starvation on the cell death rate, product stability, and up-growth of plasmid-free cells was concluded from investigation of a number of general and specific process parameters. Although the glucose uptake capacity was inhibited in all cultures performed, the up-growth of plasmid-free cells in the culture was strongly inhibited by fast oscillations. In connection to product formation the initial α-glucosidase accumulation was the same in all cultures, but the stability of the product was significantly lower in the cultivation with long cycles, possibly because of a higher stress level.

Finally, a study of cell growth kinetics and physiology during large-scale (12 m^3 / 30 m^3) fermentation of *E. coli* W3110 including a recombinant ZZ protein process was performed within a EU network project. The data obtained from the large-scale processes demonstrate the existence of gradients for glucose and oxygen and show the effect of mixing on cell growth and product formation.

Keywords: *E. coli*, recombinant protein, cell segregation, cell viability, plasmid stability, plasmid amplification, glucose uptake, stress response, cAMP, ppGpp, sigma S, energy charge, nucleotide, glucose oscillations, fed-batch fermentation, scale-down, large-scale, α-glucosidase, creatinine imino hydrolase, ZZ

Lin, Hongying (1999). **Zelluläre Reaktionen auf die Induktion rekombinanter Gene in Fed-batch Prozessen mit *Escherichia coli***. Institut für Biotechnologie der Martin-Luther-Universität Halle-Wittenberg, Deutschland

Zusammenfassung

Ziel dieser Arbeit ist die Untersuchung zellulärer Reaktionen auf die Induktion rekombinanter Gene in Fed-batch Prozessen mit *Escherichia coli*, wobei auch Einflüsse der Maßstab-vergrößerung biotechnologischer Prozesse mit rekombinanten Mikroorganismen auf die Produktivität und die Zellphysiologie berücksichtigt werden. Als Modellsystem wurde im Rahmen dieser Arbeit ein rekombinanter Fed-Batch-Prozeß zur Produktion von α-Glucosidase ausgewählt. Dieser Prozeß wurde in Bezug auf Wachstum, Zellsegregation, Plasmidstabilität, und Produktbildung charakterisiert. Darüber hinaus wurden jedoch auch Veränderungen der Substrataufnahme, des Nukleotidpools, des Proteinmusters, sowie der Einfluß der Induktion auf die Expression verschiedener mRNA's untersucht. Die am α-Glucosidase-Prozeß gewonnenen Ergebnisse wurden mit zwei weiteren rekombinanten Prozessen verglichen (Creatinin-Iminohydrolase, ZZ-Protein), um Faktoren zu evaluieren, die verschiedene rekombinante Prozesse voneinander unterscheiden.

Aus grundlagenorientierter Sicht hat die Arbeit folgende wichtige Nachweise geliefert:

1) Nach Induktion rekombinanter α-Glucosidase kommt es zu einer Inhibition verschiedener zellulärer Prozesse. Als Folge davon kommt es zu einer Deregulation der Plasmidreplikation, mit der Folge einer 3-6-fachen Plasmidamplifikation. Das Phänomen der Plasmidamplifikation nach Induktion ist nicht auf den α-Glucosidase-Prozeß beschränkt, sondern tritt in allen Systemen auf, bei denen die Produktbildung mit einer starken Inhibition des Wachstums einhergeht.

2) Die Entstehung nicht-teilungsfähiger Zellen nach Induktion der α-Glucosidase ist eine Folge der Last der Produktsynthese auf den Syntheseapparat der Zelle. Es konnte mittels Elektronenmikroskopie gezeigt werden, daß die nicht-teilungsfähigen Zellen durch eine Ausdehnung des Chromosoms gekennzeichnet sind. Diese Zellen stellen eine metabolisch absterbende Population dar, die jedoch über einen längeren Zeitraum nicht lysiert und in der über längere Zeit noch bestimmte metabolische Aktivitäten nachgewiesen werden können. In diesem Zusammenhang wird diese Population als *viable but non-culturable* (VBNC-Status) diskutiert.

3) Die starke Induktion rekombinanter Proteine führt zu einer Inhibition der Glucoseaufnahmekapazität der Zellen. Diese Eigenschaft kann in Abhängigkeit von den Prozeßbedingungen problematisch in industriellen Prozessen sein, da die im Wachstumsmedium

akkumulierende nichtmetabolisierte Glucose das Überwachsen der Kultur durch plasmidfreie nichtproduzierende Zellen begünstigt. Im Rahmen der Arbeit wurde eine *on-line* nutzbare Methode zur schnellen Bestimmung der Glucoseaufnahmekapazität entwickelt, auf deren Basis eine optimale Regelung des Glucose-Feedings möglich ist.

4) Im Rahmen der Arbeit wurden die zellulären Alarmone ppGpp, σ^S und cAMP in Abhängigkeit von der Stärke der Glucoselimitation und der Wachstumsrate gemessen. Es konnte gezeigt werden, daß nach Induktion der α-Glucosidase im Fed-Batch-Prozeß die Konzentrationen der Regulatoren der zellulären Adaptationssysteme an Glucoselimitation (ppGpp und σ^S) reduziert sind, im Vergleich zu Kulturen ohne Induktion. Nach Überexpression der α-Glucosidase kommt zu einem Abfall der zellulären Konzentrationen von ppGpp, σ^S und der extrazellulären cAMP-Konzentration. Untersuchungen, die in diesem Zusammenhang mit verschiedenen Mutanten durchgeführt wurden, lassen vermuten, daß das Absterben der Zellen nach Induktion mit der fehlenden Adaptation an die Streßbedingungen in Zusammenhang steht.

5) Im Unterschied zum α-Glucosidase-Prozeß wurde im zweiten untersuchten System zur Produktion von Creatinin-Iminohydrolase (CRIMI) keine Wachstumsinhibition und keine Plasmidamplifikation nach Induktion beobachtet, obwohl das Produkt in höherer Konzentration (>30% vom Gesamtzellprotein) als die α-Glucosidase mit höherer spezifischer Rate gebildet wurde. Weiterhin kommt es nach Überexpression von CRIMI zu einem starken Anstieg der zellulären σ^S-Konzentration und zu einer kontinuierlichen Akkumulation von cAMP im Kulturmedium. Obwohl im Rahmen der Dissertation die molekulare Basis der unterschiedlichen Reaktion beider Systeme nicht experimentell geklärt wurde, werden im Diskussionsteil der Arbeit Hypothesen im Zusammenhang mit der Konkurrenzsituation auf Ebene von Transkription und Translation zwischen Produktsynthese einerseits und zellulären Synthesen andererseits, diskutiert.

6) Mittels einer *Scale-down* Strategie wurden Zonen mit unterschiedlicher Konzentration von Glucose imitiert, die beim Up-scaling von bakteriellen Fermentationsprozessen entstehen und untersucht, in wieweit sich Oszillationen der Kohlenstoffquelle auf die mikrobielle Physiologie und die Produktbildung auswirken. Die Ergebnisse belegen, daß oszillierender Glucosehunger die Produktbildung in rekombinanten Prozessen beeinflußt. Dies betrifft sowohl die Produktausbeute, als auch die Physiologie der kultivierten Zellen. Ein unerwartetes Ergebnis der Untersuchung war, daß sich oszillierender Glucosehunger wahrscheinlich vorrangig positiv auf den Prozeßverlauf auswirkt. Möglicherweise stellen regelmäßige Oszillationen ein schwaches Streßsignal dar, das

eine entsprechende Adaptation der Zellen auslöst und sie resistenter macht gegen den starken Streß, den die Produktion des rekombinanten Produktes darstellt.

7) Im Rahmen dieser Arbeit wurden Prozeßfaktoren evaluiert, die die Maßstabsvergrößerung rekombinanter biotechnologischer Prozesse beeinflussen. Diese Studien wurden im Rahmen des Europrojektes "*Bioprocess Scale-up Strategy — based on Intergration of Microbial Physiology and Fluid Dynamics*" durchgeführt, das neben der biologischen Charakterisierung auch die Entwicklung entsprechender Simulationsprogramme auf der Grundlage von Kompartimenten und *Fluid Dynamics* in oszillierenden Umgebungen, sowie die Large-Scale-Verifizierung im nicht rekombinanten Wildtyp *E. coli* W3110 und in einem rekombinanten Prozeß (ZZT2-Protein) beinhaltet. Es konnte durch umfassende Analysen gezeigt werden, daß die Vermischung im Großreaktor, insbesondere auftretende Glucose- und Sauerstoff-Gradienten die Zellphysiologie und Produktbildung beeinflussen.

CONTENTS

Abbreviations

ADP	3´,5´-adenosine diphosphate
AMP	3´,5´-adenosine monophosphate
ATP	3´,5´-adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic 3´,5´-adenosine monophosphate
CRIMI	creatinine imino hydrolase (EC 3.5.4.21, 45 kDa)
CRP	cAMP receptor protein, also called CAP (catabolite activator protein)
EC	energy charge
HPLC	high performance liquid chromatography
IAA	β-indole acrylic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kbp	kilo base pairs
OD_{500}	optical cell density at 500 nm
ONPG	o-nitrophenyl- β-D-galactopyranoside
NADHP	nicotinamide-adenine dinucleutide
PAGE	polyacrylamid gel electrophoresis
PFR	plug flow reactor
p-NPG	p-nitrophenyl-α-D-glucopyranoside
ppGpp	guanosine 5´-diphosphate 3´-diphosphate
RBS	ribosome binding site, also called Shine-Dalgarno-Sequence
RNAP	RNA polymerase
SDS	sodium dodecyl sulfate
STR	stirred-tank reactor
VBNC	viable but non-culturable cell population (also as VNC)
ZZ	a modified domain B of staphylococcal protein A (17.7 kDa)

Nomenclature

C_{CO2in}	carbon dioxide concentration in outlet gas
C_{CO2out}	carbon dioxide concentration in inlet gas
CER	volumetric carbon dioxide evaluation rate [mmol $L^{-1}h^{-1}$]
cfu	colony forming units [mL^{-1}]
C_{O2in}	oxygen concentration in inlet gas (in %)
C_{O2out}	oxygen concentration in outlet gas
DCW	dry cell weight [g L^{-1}]
DOT	dissolved oxygen tension [%]
Fs	substrate feed rate [g h^{-1}]
H	Henry constant
k_D	specific death rate [h^{-1}]
m	maintenance coefficient [g substrate g^{-1} biomass h^{-1}]
OUR	volumetric oxygen uptake rate [mmol L^{-1} h^{-1}]
P	product concentration [g L^{-1}]
Q	outlet gas flow rate [L h^{-1}]
q_{CO2}	specific carbon dioxide evaluation rate [mmol g^{-1} biomass h^{-1}]
q_{O2}	specific oxygen uptake rate [mmol $g^{-1}h^{-1}$]
q_p	specific product formation rate [g product $g^{-1}h^{-1}$]
q_S	specific substrate consumption rate [g substrate g^{-1} biomass h^{-1}]
R	standard gas constant; = 22.4 [L mol^{-1}]
RQ	respiratory quotient [mol CO_2 mol^{-1} O_2]
S	substrate concentration [g L^{-1}]
t	cultivation time [h]
T	temperature [°C]
V	culture volume [L]
X	cell mass; dry cell weight [g L^{-1}]
$Y_{x/s}$	yield coefficient for biomass per substrate [g biomass g^{-1} biomass]
μ	specific growth rate [h^{-1}]

1 Introduction

The recent progress of genetic engineering allows the enrichment of high value therapeutics and other recombinant proteins in bacteria up to very high levels of the cell protein. However, for successful production of a protein the thoughtful integration of information from bacterial genetics, physiology, nucleic acid and protein chemistry, and biochemical engineering is required (Georgiou, 1996).

An effective industrial process is characterized by high product concentrations at a high cell mass (Riesenberg & Guttke, 1999). By the common way high cell densities are obtained with a fed-batch procedure. During the feed phase one defined medium component is continuously added to the fermenter in a growth-limiting amount in order to control the growth conditions, such as overflow metabolism, accumulation of toxic compounds and oxygen availability (Yamané and Shimizu, 1984). As the growth rate in a fed-batch culture is generally lower than the maximum growth rate of the organism, a cell which is cultivated under fed-batch conditions to high cell densities has a very different physiological and metabolic status than a cell which is grown at low density in nutrient broth in shake flasks. This difference surely influences synthesis rates, protein stability, and protein folding, and therefore it can be suggested that the process also has a major influence on the down stream purification process.

A further important parameter in the industrial production is the scale of process. Large conventional bioreactors are commonly inhomogenous systems with respect to nutrient concentrations, gas distribution, and pH profile, mainly due to mixing and mass transfer limitations caused by a realistic power input. Recent studies indicated that microorganisms react to gradients in the reactor by a short-term response which finally can influence the process (Larsson & Enfors, 1988; Neubauer et al., 1995a,b; Larsson et al., 1996; Bylund et al., 1998; Xu et al., 1999). However, the overall effect of such inhomogeneity on the process performance is still not well investigated.

The aim of this thesis was to study extensively the effects of recombinant protein production on the host cell physiology in context to the cultivation method and the production scale for one model protein, a heterologous α-glucosidase from *Saccharomyces cerevisiae*. Thereby, we concentrated on specific parameters, such as cell growth, viability, plasmid stability, product formation, and some connected cellular responses. In the case of scale effects the study was focused on the question how repeated short term glucose starvation influences the production.

The comprehensive study on this specific model protein, α-glucosidase, was for some specific questions extended to processes with two other model proteins. Although the investigations with both other proteins are comparable to the α-glucosidase process only in a limited number of parameters, the study with the three different proteins was important for the critical discussion of the influence which is caused by the specific product characteristics.

1.1 Principle aspects of recombinant gene expression in E. coli

The overexpression of heterologous genes is influenced by several factors like plasmid stability, plasmid copy number, strength of promoter, stability of mRNA, availability of ribosomes, transcription and translation efficiency, post-translational modification, the stability and solubility of the recombinant protein itself, as well as host cell and culture conditions (Sawers & Jarsch, 1996).

Recombinant processes aiming for a high amount of heterologous protein are often based on the use of strong expression systems which are regulated at the level of transcription (Swartz, 1996; Vicente et al., 1999). Therefore, strong inducible promoter are used, such as P_{lac}, λP_L, and λP_R, or the promoter of the T7 RNA polymerase (Remaut et al.,1981; DeBoer et al., 1983; Studier and Moffatt, 1986). Such systems are commonly used for transient production of the recombinant protein, which is induced after a growth phase during which product formation is low. In many cases, after performing the inducing signal the specific production rate increases to a maximum only within a short time and product synthesis continues for one to four hours. Although in most cases it is sufficient to increase the product to a high part of the cell protein, mistranslation, aborted translation, product modification, product aggregation and degradation are consequences, which could be suggested to negatively influence the down-stream purification process. Whereas optimization is mostly performed by random screening procedures, a more comprehensive knowledge about the cellular processes and regulations in inducible recombinant systems is necessary for a knowledge based optimization.

Several cellular processes have been investigated in different expression systems in connection to the question how they are influenced following induction. So high synthesis of heterologous proteins often effects the central carbon metabolism, which sometimes results in an elevated accumulation of acetate (Shimizu et al., 1988; Seeger et al., 1995). Also the respiratory activity has been described to increase after IPTG addition (Bhattacharya & Dubey, 1997), however, the interconnection between the change of the carbon metabolism and respiration has not been analyzed in detail yet.

Although one should assume a drastic change of the protein synthesis pattern after induction when the synthesis of the recombinant product occupies most of the total protein generating system, only a few articles were looking on this fact (Bailey, 1993) and a comprehensive analysis is yet missing. However, it is obvious that transcription as well as translation of the product compete with the synthesis of house-keeping proteins and decrease their synthesis within minutes after induction (Vind et al., 1993; Rinas, 1996; Dong et al., 1995). Interestingly, all three groups, although using different systems and procedures for production of their recombinant product, found a strong reduction of the synthesis rate and the concentration of ribosomal proteins.

Aside from this reduction of house-keeping proteins, recombinant protein production often also causes a heat shock like response which is possibly triggered by incorrectly folded intermediates of the product (Goff & Goldberg, 1985, 1987; Kosinski & Bailey, 1991; Kosinski et al., 1992b). Possibly the appearance of incorrectly folded intermediates is the cause that recombinant proteins are often recognized as foreign and are consequently degraded by proteases of the host, some of which belong to the heat shock regulon. In *Escherichia coli*, the protease Lon is supposed to be mainly responsible for the degradation of abnormal and heterologous proteins in the cytoplasm (Chung & Goldberg, 1981; Goldberg, 1992; Gross, 1996). In *lon* mutants the proteolysis of abnormal polypeptides could be significantly reduced (Chung & Goldberg, 1981), and correspondingly, the yield of recombinant proteins has been reported to be positively influenced in *lon* mutants (Buell et al., 1985; Surek et al., 1991). It is also supposed that the ATP-dependent Clp protease contributes to the degradation of foreign proteins. ClpP, in association with the ATPase subunit, ClpA, is responsible for the proteolysis of proteins with abnormal amino-termini (Tobias et al., 1991). It has been suggested that the ClpP protease subunit, together with its ATPase subunits ClpX and ClpA, provides an important housekeeping function in destroying mutant or partially denaturated proteins (Wawrzynow et al., 1996; Laskowska et al., 1996), and is specifically responsible for elevated protein turnover during starvation (Damerau & St John, 1993). Kandror and coworkers (Kandror et al., 1994) demonstrated that ClpP, together with the chaperones GroEL and GroES, degrade the model recombinant protein CRAG (a short-lived fusion protein). Furthermore, ClpA and ClpP of *B. subtilis* are associated with recombinant inclusion bodies (Jürgen et al., unpublished results). This suggests that also in *E. coli*, ClpP might be involved in the degradation of recombinant proteins which accumulate in inclusion bodies.

A recent study of Aris and coworker (Aris et al., 1998) indicates that recombinant protein production is also connected to the induction of the SOS response. The authors show that the heat-induced expression of recombinant genes from strong lambda P_L-P_R promoters activates the SOS system in the harboring cells, irrespective of the nature of the encoding gene (such as VP1, VP60, VIAA, TSP), but coincident with the proteolytic instability of the resulting proteins. Although the signal and the signal transduction pathway have been not characterized yet, one can suggest from this that the product synthesis has strong detrimental effects on the cell behavior.

In contrast to the heat shock response, other stress responses, such as the stringent response and the general stress response have not been shown to be induced after induction of recombinant proteins (Andersson et al., 1996). However, the study of Andersson and coworkers was not performed with a strong induction system, and the product was only accumulated to a few percent of the total cell protein. The data from Dedhia et al. (1997) and Chou et al. (1996) with mutants in major regulatory genes for these two stress responses suggest that these responses are somehow connected to the induction of recombinant proteins and might have negative effects on the product formation.

1.2 Fed-batch as the cultivation strategy

Batch, continuous and fed-batch operation are the three main modes, which are used for microbial cell cultivation. However, recombinant processes are mostly performed as fed-batch, especially in the therapeutic area, where product quality and reproducibility (GMP rules) are major requirements.

The fed-batch procedure is based on the limiting addition of a medium component, to control the growth conditions, such as overflow metabolism, accumulation of toxic compounds and oxygen availability. In principle, a fed-batch process is a batch process with the feed of a concentrated substrate, mostly glucose. After the initial concentration of glucose is consumed during the batch phase, a second process phase is following during which the feed is continuously pumped to the reactor in a way that the added amount is so low to control the growth rate and thereby ensuring aerobic conditions, which are necessary to maintain the production capacity of the microorganism. The controlled decrease of the respiratory activity below the maximum respiration rate also decreases the metabolic overflow to acetate. This overflow of acetate from the glycolysis is a result of a limited respiratory capacity and/or low activity of the tricarboxylic acid cycle in relation to the high glucose uptake (Anderson et al., 1980). If the glucose uptake would not be controlled, acetate, which only is produced when the glucose inflow exceeds the respiratory activity, would not only

inhibit growth but might also negatively effect the recombinant protein production when the level increases above 1.0 to 1.5 gL^{-1}, as shown by different authors (Shimizu et al., 1988; Jensen & Carlsen, 1990; Luli & Strohl, 1990; San et al., 1994).

From these reasons the reduction of acetate formation in *E. coli* was a major aim in bioprocess and strain engineering, whereby various strategies were proposed, such as (a) selection of a production strain with low acetate accumulation as an optimization parameter (Van De Walle & Shiloach, 1998); (b) adjustment of the medium feed rate in accordance to the oxygen transfer capacity of the reactor (Konstantinov et al., 1990a); (c) use of oxygen enriched air or pure oxygen for aeration; (d) in situ removal of acetate by perfusion systems; (e) construction of mutant strains with reduced acetate formation, e.g. *pta*$^-$ and *ack*$^-$ mutants (San et al., 1994; Bauer et al., 1990); (f) use of alternative substrates which reduce the formation of acetate, such as glycerol (Yee & Blanch, 1992; Anderson et al., 1980). An interesting approach to decrease acetate formation by increasing the respiratory activity of the cells with a concomitant higher ATP production is based on the coexpression of a hemoglobin (Kallio et al., 1994).

In most cases, acetate formation is reduced by limiting the growth by the controlled addition of the carbon source e.g. by glucose limited fed-batch strategies. In this context, Konstantinov and coworkers (1990a,b) showed that the oxidative capacity of the cell population is not a constant because the critical specific glucose uptake rate which determines the formation of acetate varies. The authors propose controlling the feeding according to a constant oxygen uptake rate, which prevents acetate formation. Considering this, it should be noticed that product formation or a switch in the environmental conditions (e.g. higher or lower temperature) influence the glucose uptake capacity and maximum respiratory activity of the cells after induction and therefore effect the metabolic flows and overflow metabolism. This was also considered by Seeger and coworkers (1995), who found a higher formation of acetate following induction of human basic fibroblast growth factor and therefore decreased the feeding rate in the production phase.

By the fed-batch procedure, cell concentrations in the range of 50 to more than 100 g L^{-1} dry mass can be reached with recombinant *E. coli* (Curless et al., 1990; Horn et al., 1996; Riesenberg, 1999). The highest cell concentration (145 g L^{-1}) in a recombinant process with high production of a recombinant periplasmic scFv has been described by Horn et al. (1996). This cell concentration is not much lower than the highest concentrations reported in the literature for non-recombinant *E. coli* and the normal fed-batch mode (148 g L^{-1} by Korz et al., 1995; 165 g L^{-1} by Rinas et al., 1995) or

in a dialysis bioreactor (190 g L^{-1} by Nakano et al., 1997), proving that cell capability for production of target proteins can be maintained to those high concentrations.

Recombinant fed-batch processes are mostly performed by a sequence of at least two different feeding regimes (Krämer, 1996). Most carbon limited fed-batch processes for the production of recombinant proteins are performed by a predetermined addition of the concentrated feeding solution to limit the growth. This addition is commonly controlled by an exponential function (Harder et al., 1994; Riesenberg et al., 1990, 1991) or by a stepwise increase to maintain a defined growth rate (e.g. Fieschko & Ritch, 1986; Lee et al., 1989). If the feed increases to a level that the DOT would fall short of a minimum DOT level (mostly 20 %) at maximum aeration and stirrer speed, the feed pump is set to a constant rate. In connection to the cellular capacity for production a growth rate in the range of 0.1 to 0.2 h^{-1} should be maintained before induction (Hellmuth et al., 1994; Flickinger & Rouse, 1993). After induction in dependence on the process another change of the feed rate might be necessary.

1.3 The scale of production

During the process development phase many recombinant processes have to be scaled-up to the 1 to 30 m^3 scale, and sometimes even larger scales are required (up to 100 m^3).

The scale-up of a microbial fed-batch process has traditionally used technical criteria as reactor geometry, power input, mixing and mass transfer (Enfor & Häggström, 1994). The traditional methods have not taken into account that the microorganisms might react to the large scale environment. However, the importance of considering the biological part of the system has become more and more clear, because especially in recombinant fermentations the results of process scale-up were not predictable in many cases (Riesenberg et al., 1990; Bylund et al., 1998).

The problem in the scale-up of biotechnical processes is assumed to be caused by the fact that a large reactor is not as homogeneous as a small reactor due to the limit in the power input and mixing. This causes mass transport limitation and gradients in the reactor, specifically for the energy substrate, gas distribution, and the pH control substance. The heterogeneity in the reactor is even more enhanced in fed-batch cultures where a high concentrated feed solution is added (e.g. 800 g L^{-1} of glucose) to avoid a high volume build up. This highly concentrated viscous feed solution has to be diluted in the reactor by a factor of several thousand to the grow limiting concentration, which is for glucose in the mg L^{-1} range (Bylund et al., 1998). As this process is limited by the

turbulence in the reactor, cells in the near of the feeding zone will be exposed to a high substrate concentration, although for a short time. On the other side, it is assumed that zones exist, in which the substrate uptake rate exceeds the mass transfer, and which therefore are characterized by starvation. Thus, cells circulating in a large production scale bioreactor are continuously exposed to varying substrate concentrations (Fig. 1.1).

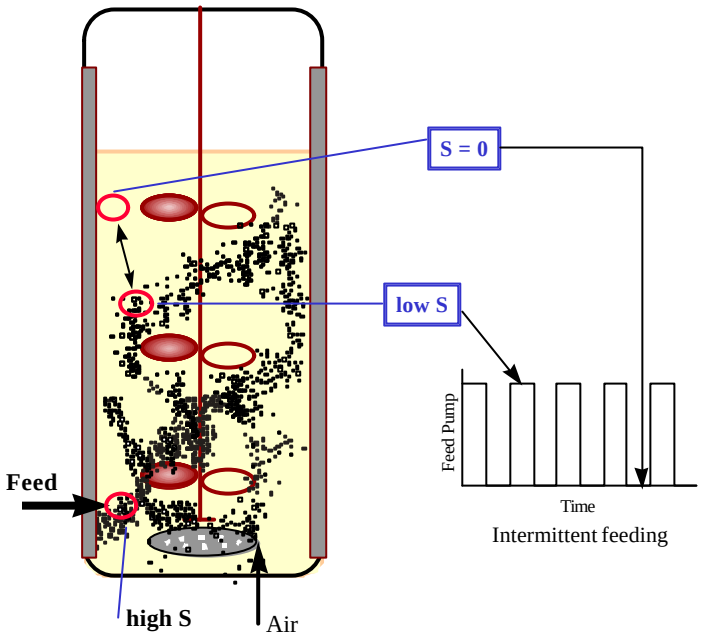


Figure 1.1: Heterogeneity in large-scale reactor during fed-batch cultivation and scale-down principle: performance of oscillations in a single reactor.

Scale-down studies in which the large scale was imitated in a small scale bioreactor indicated that microorganisms react to repeated shifts within seconds, and this response may finally also can influence the process (Namdev et al., 1993; Neubauer et al., 1995a). For example, in a two-compartment reactor system, consisting of a stirred-tank reactor (STR) and a plug-flow reactor (PFR), between which the *E. coli* cells are circulating, acetate is immediately formed as a response to the high glucose concentration in the PFR (Neubauer et al., 1995b; Xu et al., 1999). Otherwise, when the PFR was used as a glucose starvation zone, or when repeated short term glucose starvation was performed by an on/off feed procedure in a one-reactor system, a typical cellular alarmone for glucose starvation, ppGpp (guanosine 5'-diphosphate 3'-diphosphate), was immediately synthesized (Neubauer et al., 1995a). Unexpectedly, although an immediate short term response was detected in both cases, the effect on general process growth and yield parameters, such as maximum biomass formation (X_{mas}) and $Y_{x/s}$, was not significant when a wildtype *E. coli* was cultivated, but these parameters were strongly effected when yeast cultures were cultivated in the same reactor system (George et al., 1993, 1997).

Larsson and Enfors (1988) used a STR/PFR reactor system which consists of one well mixed aerobic part (STR) and one minor anaerobic part (PFR) to investigate the effect of short time oxygen starvation on the respiratory capacity in a *Penicillium chrysogenum* process. The results showed that irreversible inhibition of the respiration was caused by a residence time of 5 and 10 min in the PFR. The authors suggested that a lag phase existed in the inactivation, because short residence times of 1 and 2 min did not influence the process.

The effect of temperature oscillations on ethanol production were studied during a continuous culture of *S. cerevisiae* in a reactor equipped with a shift-up temperature loop (Groot et al. 1992). The ethanol production rate was influenced by the magnitude of temperature change (a shift-up of 5 K lowered the ethanol production) and by the time in the loop. There was no effect when the residence time in the loop was less than 18 seconds.

The biological reactions and the integrity of the cells are also suggested to be sensitive to extreme pH. In large-scale processes a considerable feed of alkali/acid is injected for pH control, mostly through one injection port, and this may impair the process when the zone of extreme pH becomes large. Also relatively small deviations from the controlled pH can be expected to have physiological effects on the cells.

Several scale-down approaches have been developed on a laboratory scale in order to reveal the nature of the response towards the dynamic conditions in a large scale bioreactor (Oosterhuis et al, 1983; Sweere et al., 1987; Larsson, 1990; George et al., 1993; Bylund et al., 1999). Principally two different strategies can be distinguished.

In *coupled bioreactors systems* the culture is circulated between a STR and a PFR or between two STR's. The cells are circulated between the two reactors, which imitate different zones of a large reactor. These methods have been extensively discussed in the PhD theses of Larsson (1990) and George (1997) and recent literatures (Larsson & Enfors, 1988; George et al., 1993; Neubauer et al., 1995a; Bylund et al., 1999).

Oscillations can also be created in a single laboratory reactor system by e.g. *repeated pulses of substrate* (I), varying the feed of oxygen (Namdev, 1993) or limiting substrate (Neubauer et al., 1995a; Törnkvist et al., 1996) (II), or by oscillation of the head pressure for gaseous substrate (Vardar & Lilly, 1982) (III). In this kind of cultures all cells are simultaneously exposed to the oscillating conditions.

In this work a single bioreactor was used with intermittent feed of glucose in order to investigate the fast response of repeated short-term glucose starvation during glucose limited fed-batch cultures of recombinant *E. coli* cells. Fig. 1.1 shows a simplified profile of this intermittent feed approach.

1.4 Objectives

The advent of recombinant DNA techniques confers on bacteria the possibility to produce novel products of high biotechnological value. The high potential lies also in the ability to grow bacteria to high cell densities and for them to produce the protein product as a large part of their final biomass. However, although the specific cellular responses of *E. coli* to starvation and stress are principally well studied, the knowledge about the cell physiology is low in connection to the responses which are triggered by the recombinant product formation. This research area is especially neglected, because of the complexity of the reactions which are influenced and the variation of the specific effects by the respective product and expression system. On the other side, it becomes more and more clear that only by understanding the cellular reactions one will come away from the actual use of empirical strategies for process design and optimization. The attempt to understand the complexity of the reactions is presently supported by a number of new powerful techniques, reaching from proteome and transcriptome analysis to metabolic flow approaches and finally to computational applications such as multivariate analysis and hybrid process modelling.

The goal of this thesis was a detailed description of the cellular reactions in response to the overexpression of heterologous genes during glucose limited fed-batch recombinant *E. coli* cultures with a focus on the cellular stress responses and the fitness of the production system, including cell viability.

In this context it has been the aim of these studies to evaluate the importance of the stringent response and the general stress response by σ^S, as both networks have been shown to play a general role in the adaptation of the *E. coli* cell to a wide range of stress and starvation conditions.

Whereas the major investigations should be performed with one model system, a process for a yeast α-glucosidase, the results should be also compared to other processes. In this connection two other recombinant products were chosen from cooperations with the pharmaceutical industry, a CRIMI (creatinine imino hydrolase) and a ZZ protein (a modified domain B of staphylococcal protein A).

Especially, following aspects were investigated:

1) Influence of recombinant α-glucosidase production on cell growth, cell segregation, plasmid stability and amplification, formation of the recombinant protein was investigated during fed-batch recombinant cultures. These results obtained from the α-glucosidase process were compared with the recombinant CRIMI overproduction in order to find out which factors play an important role on the changed cell physiology and the overproduction of recombinant protein.

2) To obtain a comprehensive picture of the cell viability, several cellular key reactions, such as replication, transcription, translation, substrate uptake and respiration were analyzed and the status of induced cells was studied. Furthermore, the maintenance of the energetic situation of the cell during the production phase was investigated by quantifying the concentration of the adenosine nucleotides such as ATP, ADP and AMP to find out how the energy regeneration is influenced by the product synthesis.

3) To investigate the effect of the overproduction of heterologous gene in *E. coli* on the level of different cellular stress response regulators, such as ppGpp, σ^S and cAMP. Meanwhile, to study which role the general stress response plays in survival of cells after induction by using *clpP*-deficient and *clpP- ropS*- deficient mutant strains. Furthermore, the influence of overexpression of recombinant genes on the concentration of these starvation signals and cAMP was compared by several different expression systems.

4) The small scale process of α-glucosidase was then investigated by a down-scale procedure using intermittent feed with a different length of the feed cycle to find out the influence of repeated short term glucose starvation on the recombinant *E. coli* process, especially on such parameters as cell death rate, product stability, and up-growth of plasmid-free cells.

5) Finally, the cell growth kinetics and physiology of *E. coli* W3110 with and without overproduction of ZZ protein were studied in large-scale fermenters within a EU network project based on integration of microbial physiology and fluid dynamics.

2 Materials and Methods

2.1 Strains and Plasmids

2.1.1 Strains

The strains used in this thesis are listed in Table 2.1. The *Escherichia coli* K-12 strains W3110 and RB791 (F^-, IN(rrnD-rrnE)1, λ^-, $lacI^qL_8$) were kindly provided by the *E. coli* Stock Center (New Haven, USA). The *E. coli* strains RB791P, RB791PS and RB791L were obtained from T. Schweder (Univ. Greifswald). They were constructed by P1 transduction of *E. coli* RB791 as described by Schweder et al. (1996).

Table 2.1: *E. coli* strains used in this work

Strain	Characteristics	Source	Reference
W3110	F^- IN(rrnD-rrnE)1 λ^-	*E. coli* Stock Center (New Haven)	Jensen, 1997; Smith & Neidhardt, 1983
RB791	as W3110, $lacI^qL_8$	*E. coli* Stock Center (New Haven)	Brent R., 1981
RB791P	RB791 *clp::cam*	T. Schweder (Univ. Greifswald)	Schweder et al., 1996
RB791PS	RB791 *clp::cam, rpoS::tc*	T. Schweder (Univ. Greifswald)	Schweder et al., 1996

The strains were stored in 20 % glycerol at -20 °C.

2.1.2 Plasmids

The plasmids used in this thesis are listed in Table 2.1 (also see Fig. 2.1). The plasmid pKK177glucC (Kopetzki et al., 1989a) is a ColE1 derivative containing the gene of the *Saccharomyces cerevisiae* α-glucosidase (EC 3.2.1.20, 67 kDa) under control of the *tac* promoter (DeBoer et al., 1983). The plasmid pDSCrimi contains a T_5-promoter which regulates the synthesis of the recombinant creatinine imino hydrolase (EC 3.5.4.21, 45 kDa). Both plasmids contain the *bla* gene for selection pressure. The plasmid pRIT44T2 is a pBR322 derivative and contains the gene for the ZZ protein (17.7 kDa), a modified domain B of staphylococcal protein A (Köhler et al., 1991), which synthesis is regulated by the indole-3-acrylic acid (IAA) inducible *trp*-promoter (Amann et al., 1983). Plasmid pUBS520 which was used in connection with pKK177glucC to increase the level of the minor *dnaY* tRNA (AGA/AGG) is a pACYC177 derivative, containing the *lacI*^{q} gene from plasmid pMC1 and a 540-bp *Dpn*I fragment from pDM201 encoding the *E. coli dnaY* gene (Brinkmann, et al., 1989).

Table 2.2: Plasmids used in this work

Plasmid	Characteristics	Products	bp	Source and reference
pKK177gluC	Amp^r, P_{tac}, rop^-	α-glucosidase from *S. cerevisiae* (67 kDa)	4675	Boehringer Mannheim (Penzberg, Germany; Kopetzki et al., 1989a)
pDSCrimi	Amp^r, P_{T5}	Creatinine imino hydrolase (45 kDa)	3816	Boehringer Mannheim (Penzberg, Germany)
pRIT44T2	Amp^r, P_{trp}	ZZ (17.7 kDa)	-	Köhler et al., 1991 (Stockholm, Sweden)
pUBS520	Km^r, $lacI^q$, *dna*Y	LacI repressor $tRNA^{Arg}_{AGA/AGG}$	5363	Boehringer Mannheim (Penzberg, Germany; Brinkmann et al., 1989)

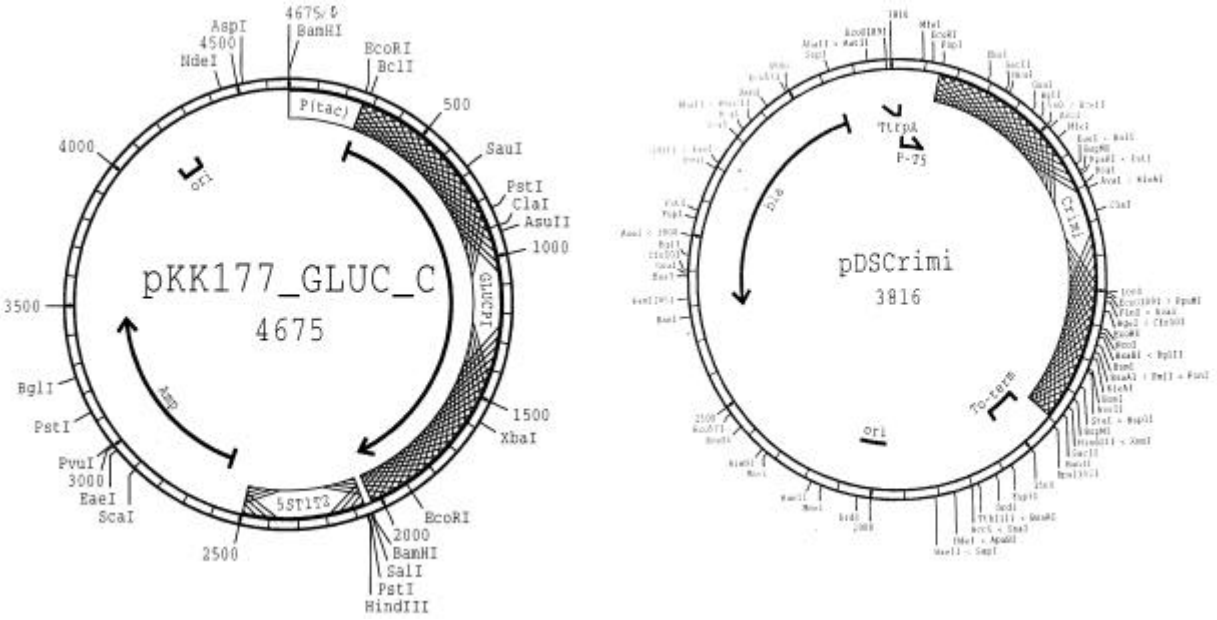


Figure 2.1: Construction map of plasmids pKK177GlucC and pDSCrimi.

2.2 Cultivation media and conditions

2.2.1 Cultivation medium

Complex medium. Nutrient Broth in double concentration (NB II, Difco) was mixed with de-ionized water and autoclaved at 121 °C for 30 min.

Mineral salt medium (MSM). Glucose-ammonia based mineral salt medium with the following composition was used in all cultivations (in $g\,L^{-1}$): Na_2SO_4 2.0, $(NH_4)_2SO_4$ 2.47, NH_4Cl 0.5, K_2HPO_4 14.6, $NaH_2PO_4 \times 2\,H_2O$ 4.0, $(NH_4)_2$-H-citrate 1.0, and thiamine 0.1. The medium also contained 1M $MgSO_4$ (2 $mL\,L^{-1}$) and 3 $mL\,L^{-1}$ of trace component solution (Table 2.3, Holme et al., 1970). The initial glucose concentration was 5 $g\,L^{-1}$ in all fermentations, but 10 $g\,L^{-1}$ in shake flask cultivations. The feed solution for the fed-batch cultivations contained (in $g\,kg^{-1}$): glucose 200, Na_2SO_4 2.0, $(NH_4)_2SO_4$ 2.0, NH_4Cl 0.5, K_2HPO_4 14.6, $NaH_2PO_4 \times 2H_2O$ 4.0, $(NH_4)_2$-H-citrate

1.0, and trace component solution 10 mL kg^{-1}. Additional 2.75 mL L^{-1} $MgSO_4$ (1 M) was added during the fed-batch cultivations twice during the feeding to avoid precipitation.

Appropriate antibiotics for initial selection pressure were added to all agar plates, and at the start of all shake flask and fermenter cultivations (appropriate concentrations: ampicilline 100 mg L^{-1}, kanamycine 35 mg L^{-1}, chloramphenicol 10 mg L^{-1}, tetracycline 10 mg L^{-1}).

Table 2.3: Trace element solution

Chemical	Concentration [g L^{-1}]
$CaCl_2 \cdot H_2O$	0.50
$ZnSO_4 \cdot 7H_2O$	0.18
$MnSO_4 \cdot H_2O$	0.10
Na_2EDTA	20.1
$FeCl_2 \cdot 6H_2O$	16.7
$CuSO_4 \cdot 5H_2O$	0.16
$CoCl_2 \cdot 6H_2O$	0.18

2.2.2 Shake flask cultivation

Shake flask cultures were carried out in 1 L erlenmeyer shake flask with baffles containing 200 mL mineral salt medium at 37 °C and 160 rpm in a rotary shaker.

2.2.3 Laboratory scale cultivation

For pre-cultivation a single colony from a selective plate was inoculated to a 100 mL shake flask containing 10 mL NB II. The fermentation inoculum was obtained from a second 1 L flask containing mineral salt medium which was inoculated with the first preculture. Both precultures were harvested in the exponential growth phase after cultivation for approximately 8 to 12 hours at 37 °C and 160 rpm.

The experiments were carried out in a 6 L Biostat ED Bioreactor with a digital measurement and control unit (DCU) and the *MFCS* supervising system from B. Braun Biotech (Melsungen, Germany). The cultivations were started as batch cultures with an initial culture volume of 4 L at a temperature of 35 °C. Air flow rate (0.02 to 2 vvm) and stirrer speed (200 to 800 rpm) were controlled in a cascade manner at a constant DOT of 20 % during the batch phase (100 % DOT was calibrated at 2 vvm and 800 rpm). Air flow rate was set to 2 vvm at the point of feed start and stirrer speed to 800 rpm. 25 % ammonia solution was added to keep the pH above 7.0. Polypropylenglycol (PEG, 50 %, Roth) was added as antifoam agent by an antifoam electrode. After an initial batch phase to about 1.8 g DCW L^{-1} the feeding pump was started at a rate of 53.2 mg h^{-1} (2.6 g glucose L^{-1} h^{-1}). The expression of recombinant genes (α-glucosidase, CRIMI) was induced

by injection of IPTG to final concentration of 1 mM to the fermenter at the point of 3 hours after feeding start and the fermentation was followed to approximately 20 hours after induction.

2.2.4 Industrial scale cultivation

Large-scale experiments were performed in a 30 m^3-fermenter (Biocentrum, Stavanger, Norway) and in a 12 m^3-fermenter (Pharmacia & Upjohn, Strängnäs, Sweden). Recombinant protein ZZ was produced in the 12 m^3 fermenter, while the 30 m^3 fermenter (Chemap, Männedorf, Switzerland) was used for a wildtype *E. coli* W3110 process. A short summary of these large-scale experiments is given in Table 2.4. Further information can be found in the EU-project report (Blomsten, 1999). The overall fermenter geometry for both the 30 m^3 and the 12 m^3 fermenter is described in Table 2.5.

Table 2.4: Short summary of large-scale experiments

Fermentation no.	Fermenter and place	Product	Impeller configuration	Initial glucose
ET01	30 m^3; Stavanger	Biomass	Rushton	0.5 g L-1
ET02	30 m^3; Stavanger	Biomass	Scaba	0.5 g L^{-1}
ET03	30 m^3; Stavanger	Biomass	Scaba	0.5 g L^{-1}
PU01	12 m^3; Strängnäs	ZZ	Rushton	0.5 g L^{-1}
PU02	12 m^3; Strängnäs	ZZ	Scaba	5 g L^{-1}

Table 2.5: The 30 m^3 and the 12 m^3 fermenter geometry.

	30 m^3 fermenter	12 m^3 fermenter
Fermenter diameter	2.09 m	1.88 m
Fermenter height	9.60 m	4.70 m
Baffle width	0.17 m	0.21 m
Spacing baffle - wall	0.03 m	0.03 m
Baffle height	7.40 m	3.60 m
Sparger diameter	0.90 m	0.53 m
Shaft diameter	0.125 m	-
Distance bottom - sparger	0.54 m	-
Distance sparger - impeller	0.58 m	0.29 m

In cultivation ET01 to ET03 the initial medium volume was 20 m^3 and it was kept at about 22 m^3 volume by intermittent withdrawal of broth during the cultivations. The initial glucose concentration was listed in Table 2.4 and the glucose feed (454 kg m^3) was started at one hour after fermentation start. The feed rate was increased stepwise every 0.5 h in an exponential mode to give a specific growth rate of about 0.3 h^{-1} until the cell density was about 9 gL^{-1}. The actual feeding rate F(t) was calculated by the expression:

$$F(t) = \mu/Y_{x/s} \times X \times V/S_{feed} \times \exp[\mu \times (t-t_0)] \quad (1)$$

where μ is the desired specific growth rate (h^{-1}), $Y_{X/S}$ is the cell yield on glucose (g g^{-1}), X is the cell concentration, V is the culture volume (L), S_{feed} is the concentration of glucose in the feed (g L^{-1}), and t_0 is the time at which the feed is started. After 9 h of cultivation the feeding rate was set to a constant value of 180 L h^{-1}, and a constant feed of $MgSO_4 \cdot 7H_2O$ (1 M) was started with a rate of 0.14 kg m^{-3} h^{-1}. pH was kept 7.0 by titration with 25 % ammonia.

The same fermentation procedure was performed in the cultivations PU01 and PU02 with an initial working volume of 6m^3. Ampicilline (100 mg L^{-1}) and tryptophan (100 mg L^{-1}) were added to fermenter before inoculation. The production of ZZ was induced by addition of β-indole acrylic acid (IAA) to a final concentration of 25 mg L^{-1}.

2.3 Analytical methods

2.3.1 Cell concentration

Optical density (OD). Growth of the cultures was followed by measuring the optical density at 500 nm by means of a spectrophotometer (Ultrospec 3000, Phamacia, Sweden). NaCl solution [0.9 % (w/v)] was used as reference. If the absorbance exceeds 0.6 the sample was diluted with 0.9 % (w/v) NaCl solution.

Dry cell weight and wet cell weight. Dry cell weight (DCW) was measured gravimetrically by centrifuging 4×1.5 mL of cell suspensions. Therefore, 1.5 mL cell suspensions were centrifuged in pre-weighted 2 mL eppendorf tubes and washed once with 0.9 % NaCl solution. After removal of the supernatant the samples were dried to constancy at 60 °C for at least 24 h. In average a DCW of 1 g L^{-1} resulted in an OD_{500} of 4.5±0.1, while 1 g L^{-1} wet cell weight resulted in an OD_{500} of 1±0.1.

Cell number. The samples were diluted in 0.9 % NaCl solution according to the value of OD_{500}, and a small volume (2 to 10 μL) was transferred to a counting chamber (0.02 mm depth, Neubauer, Germany). The number of cells is counted at 400 times magnification in a light microscope. Generally, a DCW of 1 g L^{-1} resulted in an *E. coli* cell number of $(2\pm0.1)\times10^9$ mL^{-1} during exponential growth phase.

Colony forming units (cfu). Colony forming units were analyzed by plating at least three dilutions and each of these at triples on NB II plates, which were incubated for one to three days at 37 °C.

The subpopulation of plasmid-carrying part was determined by replica plating from NBII-plates to antibiotic containing plates.

2.3.2 Analysis of medium compounds

2.3.2.1 Glucose concentration

The glucose concentration was estimated by the hexokinase/glucose-6-phosphate-dehydrogenase method (Kit No. 139106, Boehringer Mannheim GmbH, Germany) in microtiter plates with four parallels for each sample. Figure 2.2 shows a typical standard curve for analysis of glucose concentration.

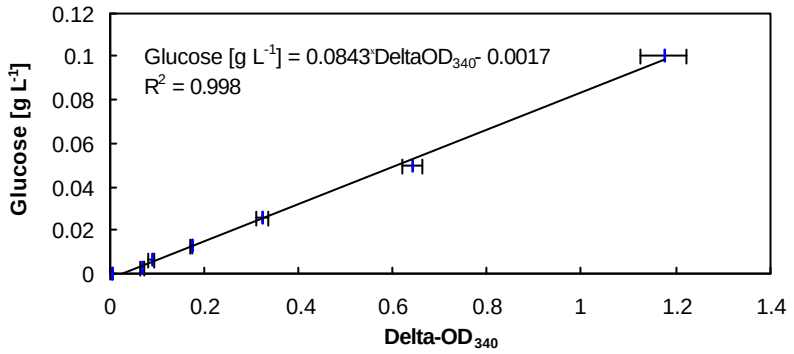


Figure 2.2: A typical standard curve of glucose concentration according to the hexokinase /glucose-6-phosphate-dehydrogenase method.

2.3.2.2 Acetate concentration

Acetate was determined enzymatically with test kit no. 148261 from Boehringer Mannheim (Germany) in microtiter plates with four parallels for each sample. A typical standard curve of acetate concentration is shown in Fig. 2.3.

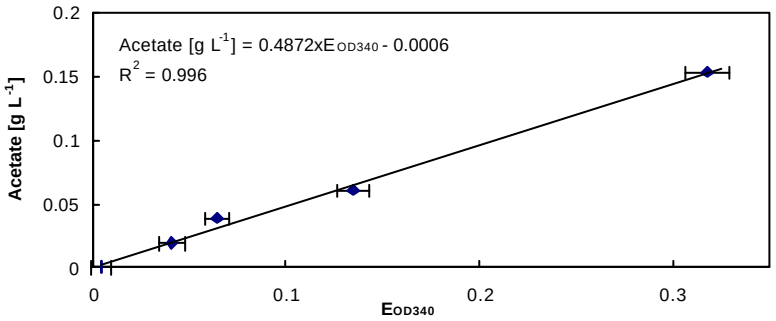


Figure 2.3: A typical standard curve of acetate concentration.

2.3.2.3 Ammonia concentration

Ammonia concentration was analyzed by an ammoniac electrode (Microprozessor pH/ION Meter pMX 3000, WTW, Weilheim, Germany) with a sample volume of 5 mL by addition of 50 µL NaOH (10 M). If $[NH_4]^+$ concentration exceeded 200 mg L^{-1}, the sample was diluted with deionized water.

2.3.3 Enzyme assays

2.3.3.1 a -glucosidase activity

Soluble α-glucosidase was extracted after cell disruption with fine glass beads (2.5-5 mm) in 10 mM potassium phosphate buffer (pH 6.8) with 1 mM EDTA as described in section 2.3.5.1. Activity of α-glucosidase was assayed at 30 °C in 0.1 M phosphate buffer (pH 6.8) and 2 mM p-nitrophenyl-α-D-glucopyranoside (p-NPG, Sigma) as substrate (Zimmermann, 1974; Stempfer, 1995). The reaction was started by addition of 100 µl p-NPG (20 mM). The release of p-nitrophenyl from hydrolysis of p-NPG was followed by measuring the rate of increased absorbance at 405 nm (ΔE_{405} min^{-1}) with spectrophotometer DU 640 (Beckman).

The enzyme activity was calculated according to the following formula.

$$\text{activity [U mL}^{-1}] = \frac{V_{test} \times \Delta E_{405}/\text{min}}{\varepsilon_{405nm} \times d \times V_{sample}} \times \frac{V_{cell\ extract}}{V_{culture}} \qquad (2)$$

$$\text{specific activity [U mg}^{-1}\text{ DCW]} = \text{activity [U mL}^{-1}] / x \qquad (3)$$

$$\text{specific activity [U mg}^{-1}\text{ protein]} = \text{activity [U mL}^{-1}] / c \qquad (4)$$

ΔE_{405}/min:	increased absorbance per minute at 405 nm
V_{test} :	total test volume in cuvette (= 1 mL)
ε_{405nm} :	absorbance coefficient, $\varepsilon_{p\text{-nitrophenol}}$ = 7.8 µmol^{-1} cm^{-1} (pH 6.8)
d:	length of cuvette (= 1cm)
V_{sample} :	volume of added sample [mL]
$V_{cell\ extract}$	cell extract volume (= 800 µL)
$V_{culture}$	culture volume for getting a pelleted cell sample of 20 mL according to OD_{500}=1
c:	protein concentration [mg mL^{-1}]
x:	dry cell weight [g L^{-1}]

Definition: 1 Unit = 1µmol p-nitrophenol formation min^{-1} (5)

2.3.3.2 Creatinine imino hydrolase activity

Activity of creatinine imino hydrolase (CRIMI) was assayed at 30 °C in 0.2 M sodium phosphate buffer and creatinine as substrate according to the method described by Boehringer Penzberg. The test principle is based on the following reactions.

$$\text{Creatinine} + H_2O \xrightarrow{\text{CRIMI}} \text{N-Methylhydantoine} + NH_3 \quad (6)$$

$$\text{N-Methylhydantoine} + \text{ATP} + 2H_2O \xrightarrow{\text{NMHase, } Mg^{2+};\ NH_4} \text{N-Carbamoyl-Sarcosine} + \text{ADP} + \text{Pi} \quad (7)$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK; } Mg^{2+}} \text{ATP} + \text{Pyruvate} \quad (8)$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD} \quad (9)$$

The enzymatic activity was followed by measuring the rate of decreased absorbance at 365 nm (ΔE_{365} /min) due to the formation of NAD ($\varepsilon_{365nm} = 3.4\ \mu mol^{-1}\ cm^{-1}$) with spectrophotometer DU 640 (Beckman). The analysis was performed at a wavelength of 365 nm with a total test volume of 1.565 mL at 25°C. The width of used cuvette is 1 cm.

Table 2.6: Buffer composition and analytical procedure for CRIMI activity assay.

	solution [mM]	test volume [mL]	reference [mL]
TES-buffer	0.15 M	1.25	1.25
ATP	33 mM	0.1	0.1
NADH	14 mM	0.05	0.05
PEP	21.5 mM	0.05	0.05
NMHase	40 U/ml	0.005	0.005
mixing and 1-2 min incubation, then addition of			
PK/LDH		0.01	0.01
reference charge		-	0.050
sample		0.050	-
($\Delta E_{365}/min_{Prereaction}$); reaction was started by addition of			
creatinine	10 %	0.05	0.05
mixing, ($\Delta E_{365}/min_{Sample}$)			

The enzyme activity was calculated according to following formula:

$$\Delta E_{365}/\text{min} = [\Delta E_{365}/\text{min}]_{Sample} - [\Delta E_{365}/\text{min}]_{Prereaction} \quad (10)$$

$$\text{test activity } [U\ mL^{-1}] = [1.565 / (\varepsilon_{365nm} \times 0.05 \times 1)] \times [\Delta E_{365}/\text{min}]\ [U\ mL^{-1}] \quad (11)$$

$$\text{real activity } [U\ mg^{-1}] = \text{test activity}_{Sample} \times (82.8/\ \text{test activity}_{Reference}) \quad (12)$$

where the activity of reference was 82.8 U mg^{-1}.

2.3.4 Preparation and quantification of DNA and RNA

2.3.4.1 DNA agarose gel electrophoresis

Plasmid DNA and chromosomal DNA was separated on 0.8 % (w/v) or 0.5 % (w/v) agarose gels at 4 °C in TAE buffer (40 mM Tris/HCl, pH 8.2; 2 mM sodium acetate; 4 mM EDTA). The gel was

stained with an ethidium bromide solution (1µg mL^{-1}) and the DNA was visualized on a transilluminator.

2.3.4.2 Plasmid purification and quantification

Plasmid DNA was isolated using a Qiagen plasmid purification kit (kit no. 20 104, Qiagen). Plasmid purity was controlled by agarose gel electrophoresis.

Plasmid concentration was calculated based on the absorbance at 260 nm (E_{260}) with a GeneQuant RNA/DNA Calculator (Pharmacia, Sweden) and related to the cell amount. The plasmid copy number is defined as the number of plasmid copies per cell and is based on the calculation of the molecular weight of the plasmid (average molecular weight of a base is 309 g mol^{-1}, Neidhardt et al., 1990) relative to the cell number estimated by microscopy. The plasmid DNA purity was checked by the ratio of $E_{260/280}$ which should be between 1.65 to 1.71 and by analysis of the sample on agarose gels.

plasmid content c_{P1}	[µg mL^{-1} culture broth]	$= (\varepsilon_{DNA} \times E_{260} \times V_{Eluate}) / (5/OD_{500})$	(13)
specific plasmid content c_{P2}	[µg mg^{-1} DCW]	$= c_{P1} / X$	(14)
plasmid copy number c_{P3}	[number per cell]	$= (c_{P1} / MW_{plasmid}) / N_{cell}$	(15)

ε_{DNA}	constant of DNA concentration	= 50 µg $µL^{-1}$
E_{260}	absorbance at 260 nm	
V_{Eluate}	amount of plasmid elution by Qiagen	= 50 µL or 100 µL
X	dry cell weight [mg mL^{-1}]	
$MW_{plasmid}$	weight of one plasmid (see Table 2.7)	
N_{cell}	cell number [number mL^{-1}]	

Table 2.7: Data of plasmid weight

	bp	size of one plasmid strand [Da][a]	weight per plasmid
pKK177glucC	4675	1.44×10^{6}	4.78×10^{-12} µg
pDSCrimi	3816	1.18×10^{6}	3.92×10^{-12} µg

[a]: 1 atom unit mass [Da] = 1.66×10^{-24} g (Stryer, 1996)

2.3.4.3 Cell transformation

An overnight culture of *E. coli* RB791 was diluted 1/100 with 50 mL of fresh NB II medium and incubated in a shaker at 37 °C for 3 to 4 h until the culture reached an optical density at 500 nm of 0.3 to 0.5. The culture was centrifuged at 8000 rpm for 10 min at 4 °C. The cells were resuspended in 50 mL chilled transformation buffer (100 mM $CaCl_2$, 10 mM RbCl, 5 mM Tris/HCl, pH 7,5) and placed on ice for 45 min. Cells were again centrifuged as above, the pellet was resuspended in 2 mL of the same buffer and placed on ice for 2 h, of which 200 µL were used for transformation. The competent cells could be stored at -70 °C up to 6 months when 20 % glycerol was added.

About 1-10 ng plasmid DNA or 10-100 ng DNA ligation sample was added to 200 µL of competent cells and the mixture was chilled on ice for 30 min, after which it received a 90 sec, 42 °C heat pulse. The pulse was terminated with the addition of 1 mL NBII medium. The cells were grown for 1 h at 37 °C and plated on selective media. The plates were incubated for 1 to 3 days at 37 °C.

2.3.4.4 mRNA analysis

Sample and RNA isolation. Cells according to 0.5 to 1.0 mg dry weight were harvested into 400 µL of ice-cold "killing-buffer" (20 mM NaN_3, 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5) and centrifuged (3 min, 13000 rpm, 4 °C). The supernatant was removed and the pellets were frozen at -20 °C for further analysis. Total cellular RNA was isolated with the High pure RNA isolation kit (Boehringer Mannheim) according to the manufacturers instruction.

Slot-Blot-Hybridization*. The isolated RNA was diluted with 10×SSC (1.5 M NaCl, 0.15 M Na-citrate, pH 7) and transferred in different dilutions onto a positively charged nylon membrane in a slot blot apparatus, followed by hybridization with digoxigenin-labelled RNA probes. These RNA probes were synthesized in vitro with T7 RNA polymerase from PCR products containing a T7 promoter sequence. The following primers have been used for synthesis of the appropriate PCR products:

5´CAGTGATAACGATTTGGCCG and
5´CTAATACGACTCACTATAGGGAGAGTTGCGTATGTTGAGAAGCG for *rpoS*,
5´GATCCTCGAAGACCAATCCG and
5´CTAATACGACTCACTATAGGAGAGAATCTGGTAAACCAGGACG for *glucC*,
5´GGGTAAAATAATGGTATCG and
5´CTAATACGACTCACTATAGGGAGACTTTGATGTTCATGTGTTTC for *dnaK*,
5´TGCTGGCTGTAATGTTGACC and
5´CTAATACGACTCACTATAGGGAGACCTGGCTTTCAACGAAACCG for *osmY*,
5´GACCGAGGTTGAGATTGATGG and
5´CTAATACGACTCACTATAGGGAGACGCGCTGAACTTTGATACGC for *rpoA*.

The hybridization signals on the filters were quantified with the Lumi-Imager from Boehringer Mannheim (Germany). The signals were calculated to the amount of total RNA in the samples. For graphic presentation of the mRNA levels in this study the closest sample shortly to the point of

* This analysis of Slot-Blot-Hybridisation was performed by Britta Jürgen at the University of Greifswald.

induction was set to one. All the other mRNA levels were calculated according to the value of this sample.

2.3.5 Protein preparation and quantification

2.3.5.1 Cell disruption in a cell mill

Recombinant protein can be formed either in soluble form which is enzymatically active, or in inclusion bodies (IB's). The soluble fraction was analyzed from a pelleted cell sample of 20 mL according to OD_{500}=1. For the analysis of the activity or concentration of soluble α-glucosidase or CRIMI the pellets were resuspended in 350 µL of 10 mM phosphate buffer (pH 6.8) with 1 mM EDTA, and disrupted using a cell mill (Retsch, Germany, 5 min, 80 %) by addition of 0.5 g of glass beads (diameter 0.25 to 0.5 mm). Following, another 450 µL of 10 mM phosphate buffer (pH 6.8) was added to get a cell extract of a 800 µL total volume. After centrifugation (13000 rpm, 10 min), the soluble active recombinant proteins in the supernatant were used for activity assays (see section 2.3.3).

2.3.5.2 Preparation of inclusion bodies (IB's)

The cell pellets containing the insoluble proteins from section 2.3.5.1 were further desintegrated by addition of 475 µL Tris buffer (100 mM Tris-HCl, 1 mM EDTA) and 37 µL lysozyme (7.5 g L^{-1}) according to the method described by Rudolph et al. (1996). After incubation at 0 °C for 30 min the sample was treated by a cell mill for a second time (5 min, 80 %). The pellet was incubated for 45 min at room temperature with 37 µL of 90 mM $MgCl_2$ and 1.5 µL of DNase (Benzonase, no. 105 9949, Merck, Germany). After addition of 370 µL of an EDTA-Triton X100 solution (60 mM EDTA, 1.5 M NaCl, 6 % Triton X100) the sample was incubated for 30 min at 0°C again and then centrifuged at 13000 rpm for 10 min at 4 °C. The pellet was washed twice with 1 mL of ice-cold buffer (100 mM Tris, 20 mM EDTA). Further, the pellets were resolved by boiling for 5 min in 300 to 500 µL of sample buffer (see section 2.3.5.3). Afterwards, the IB-samples were separated on 7 % SDS-gels.

2.3.5.3 Quantification of protein on SDS-gels

For the determination of recombinant protein concentration as a fraction of the total cellular protein, samples corresponding to 1 mL of OD_{500}=1 were boiled for 10 min in 50 µL sample buffer (4 % (w/v) SDS, 20 % (w/v) glycerol, 5 % (w/v) mercaptoethanol, 0.1 % bromphenolblue, and 90 mM

Tris/HCl (pH 6.8)). The samples for total cellular protein were separated on SDS-PAGE with 5 % stacking gel and 10 % separation gel in a Mighty Small chamber (Phamacia, Sweden) according to the method described by Laemmli (1970). The gel run at 160 V for 1 to 2 h at 4°C. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (40 % methanol, 10 % acetic acid and 0.2 % coomassie blue), destained with (40 % methanol, 10 % acetic acid), enclosed in a cellophane foil and dried at 80°C for 3 hours. Afterwards, all bands of each slot were scanned by a Sharp Scanner (Japan) and the band intensity was quantitatively analyzed with Phoretix 1D software (Phoretix, England) by comparison to a standard of the corresponding recombinant protein which was added at different concentrations on each gel. Fig. 2.4 shows a typical standard curve of α-glucosidase content.

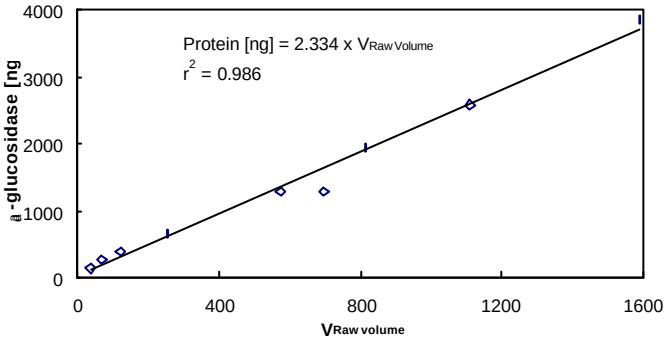


Figure 2.4: A typical α-glucosidase standard curve.

2.3.5.4 Protein quantification according to Bradford

Extracellular protein was analyzed according to the method described by Bradford (1976). Briefly, 100 µL sample was mixed with 900 µL reagent solution [containing 100 mg L^{-1} Coomassie-Brilliant-Blue G-250, 50 mL L^{-1} ethanol (95 %), 100 mL L^{-1} phosphoric acid (85 % w/v), filtered] and incubated for 5 min at room temperature. The absorbance at 595 nm was measured. Fig. 2.5 shows a typical standard curve with bovine serum albumin (BSA) as a standard.

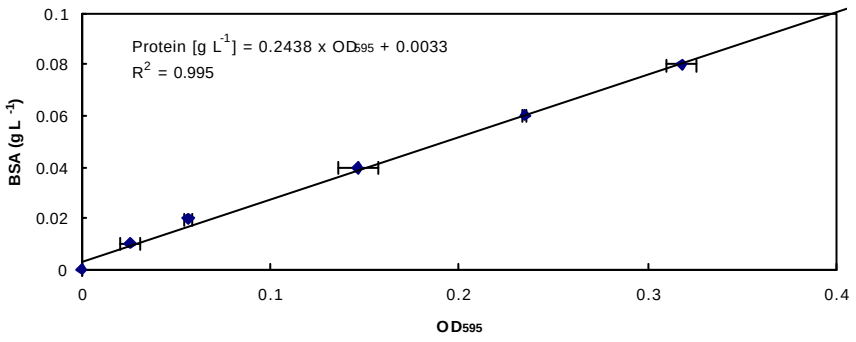


Figure 2.5: A typical standard curve of protein content according to Bradford (1976).

2.3.6 Protein analysis by immunoblot

2.3.6.1 Analysis of σ^S concentration

The concentration of σ^S was analyzed by an immunoblot method (Burnette, 1981; Lange et al., 1994). A cell concentration of about 0.28 mg DCW corresponding to 1 mL broth at OD_{500} = 1 was collected by centrifugation (5 min, 13000 rpm, 4 °C). Pellets were washed with 0.9 % (w/v) NaCl and stored at -20 °C for further analysis.

The cell samples were resuspended in 50 µL 1xSDS-PAGE sample buffer and boiled for 5min. This solution (10-25 µL) was applied to each slot of a 12 % SDS-gel. After separation about 1 h at 160 V, the gel was directly electroblotted onto a PVDF membrane (Lot No.144595A, Bio-Rad Laboratories, Hercules, U.S.) for 1 h at 100 mA and 10 V by using a Trans-Blot Semi-Dry Transfer cell (BIO-RAD). The membrane was immediately blocked with TBT buffer (10 mM Tris/HCl, 150 mM NaCl, 0.2 % Tween 20, pH 7.5) containing 5 % (w/v) skimmed milk powder and 0.05 % sodium azide for 3 h or overnight. The blots were decorated with the polyclonal antiserum against σ^S (provided by R. Hengge-Aronis, Berlin) overnight under gentle agitation, and washed three times with TBT and once with water. Finally, the blots were incubated in TBT buffer containing 2% skimmed milk powder and a 1/100 dilution of goat anti-rabbit IgG peroxide conjugate (Sigma, Cat. No. A-6154) at room temperature for 3 hours. After four further washing steps (three times in 1xTBT and once in water) the blot was developed with Sigma Fast DAB (3'3'-diaminobenzidin-tetrahydrochloride) and Metal enhancer ($CoCl_2$, Cat. No. D-0426, Sigma). The band intensity was semiquantitatively analyzed after scanning with Phoretix 1D software. For all graphic presentations the σ^S concentration at the point of induction was set to 100 %.

2.3.6.2 Analysis of H-NS concentration

The concentration of H-NS was analyzed by the immunoblot method described in section 2.3.6.1 with the polyclonal antiserum against H-NS (provided by E. Bremer, Marburg) as the first antibody. As a positive and negative control for the H-NS analysis MC4100 (hns^+) and PD32 (hns^-) have been used, which were kindly provided by E. Bremer.

2.3.6.3 Analysis of LexA concentration†

The immunoblot method was also used for LexA quantification. However, the primary antibody was the polyclonal antiserum against LexA provided by J. W. Roberts (Univ. Cornell, Ithaca, U.S.A.),

and the bands for LexA was visualized by autoradiography with ECL solution (Amersham, cat. no. 1059243 ECL1; cat. no. 059250 ECL2).

2.3.6.4 Analysis of ribosomal protein S8 concentration[‡]

Ribosomal protein S8 was quantified by a similar immunoblot method described in section 2.3.6.1. The samples were separated on a 15 % SDS-PAGE due to the small size of the protein (13.996 kD). The SDS gel was blotted onto a nitrocellulose membrane (1 h, 100 V, 75 mA). After blocking with TBS buffer the membrane was decorated with polyclonal antibodies from sheep (provided by Dr. Brimacombe, Berlin, Germany) and incubated at 10 °C overnight. Anti-sheep IgG (Sigma, no. A-3415) was used as the secundary antibody and after repeated washing the blot was visualized by autoradiography with ECL solution.

2.3.7 Determination of nucleotide concentration by HPLC

2.3.7.1 HPLC configuration

HPLC devices used were from BECKMANN and GYNKOTEK, respectively, and consisted of pump modul, autoinjector, column oven, diode array detector and PC with control and integration software. The stationary phase was a C-18-RP-column (4.6 x 150 mm, 3 µm particle size) combined with the suitable guard column (5 µm particle size, SUPELCOSIL LC-18-T or SupelGuard, all from Supelco SA, Gland, Switzerland). The separation was performed at a flow rate of 1.5 mL min^{-1} and a column temperature of 20 °C. The detection wave lengths were 254 nm and 280 nm. Spectra were recorded from 200 to 500 nm, reference wavelength was set to 600 nm. After 15-20 runs the column was washed with several H_2O/methanol gradients.

2.3.7.2 Nucleotide (AXP) analysis

Sample preparation. Nucleotide analysis was performed according to Meyer et al. (1999). Briefly, the sampling tubes were filled with 1 mL 35 % (w/v) $HClO_4$ and cooled at -70 °C. The weight of the $HClO_4$ containing tube was determined prior to sampling. 3-4 mL culture broth were added to the tube and mixed rapidly with the frozen acid. The mixture was than rapidly frozen at -20 °C in a cryostat or cooling mixture. During the first thawing the weight of the tube was determined again (the difference of the weights gave the exact sample volume). The freeze-thaw-cycle was repeated twice. The solution was neutralized with 2 N KOH containing 0.5 M imidazole (the volume was noted).

[†] This LexA-protein analysis was performed by Silke Nicklisch.

Cells and precipitate were removed by centrifugation, the supernatant was used for nucleotide analysis by HPLC.

Analysis by HPLC. AXP was analyzed on a GYNKOTEK HPLC with Chromeleon System. The buffer for the isocratic run was prepared as follows: 2.04 mL concentrated H_3PO_4 and 10 mL 0.5 M $MgSO_4$ were mixed with 900 mL deionized water and the pH was adjusted to 6.5 with triethylamine (about 6 mL). Water was added to a final volume of 1 L (Folley et al., 1983). Before applying to the HPLC the buffer was filtrated. The column was equilibrated at least 30 min before the first measurement or after the washing step, respectively.

The stock solutions of AMP, ADP and ATP in water were used for calibration, which contained 1 mg mL^{-1} of each component, corresponding to 2.52 mM, 2.05 mM and 1.65 mM respectively. 10 µL of each stock solution were diluted with 970 µL water and measured as standard. In the same way it was done for ppGpp. Peak identity was determined on the basis of retention time and spectra.

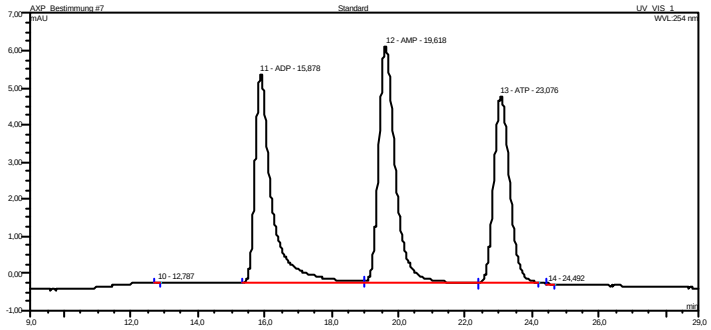


Figure 2.6: A typical chromatogram of AXP standard obtained with HPLC.

2.3.7.3 ppGpp analysis

Sample preparation. A syringe was loaded with 130 µL formaldehyde (15 %) and cooled at -70 °C. For faster cooling of the sample the syringe contained 10-15 steel beads (3 mm diameter). 4-6 mL culture broth were sucked into the syringe and rapidly mixed with the formaldehyde. The solution was aliquoted in tubes according to the OD_{500} of the culture at sampling time (1.5 mL when OD_{500} less than 10; 1 mL when OD_{500} between 10 and; 0.5 mL when OD_{500} between 20 and 50, and 0.25 mL when OD_{500} higher than 50). The aliquots were immediately centrifuged (13000 rpm, 4 °C, 3 min) and the supernatant was discarded. The pellets were stored at -70 °C until analysis.

‡ This S8-protein analysis was performed by Stephan Riemschneider.

Prior to HPLC the pellets were resuspended in 0.1 N KOH (125 µL mg^{-1} DCW) and incubated on ice for 30 min. Afterwards the solution was neutralized by adding diluted phosphoric acid (100 µL 85 % (w/v) H_3PO_4 were diluted with 9.9 mL HPLC buffer) and centrifuged. The supernatant was used for ppGpp analysis by HPLC.

Analysis by HPLC. ppGpp was analyzed on a GYNKOTEK HPLC with Chromeleon System or a BECKMANN HPLC with Gold System. The buffer for the isocratic run was prepared as follows: 17 g KH_2PO_4 and 3 g KOH were dissolved in 900 mL deionized water. 60 mL methanol, 10 mL of 1 M tetrabutylammonium dihydrogenphosphate and deionized water were added to give a final volume of 1 L (Chesbro, 1990). Before applying to the HPLC the buffer was filtrated. The column was equilibrated at least 30 min before the first measurement or after the washing step, respectively. 60 µL of standard or sample were injected.

2.3.7.4 cAMP analysis

Quantitative cAMP analysis was performed according to the isocratic HPLC method which was described above for ppGpp analysis. For calibration a 100 µM solutions of cAMP was used. 20 µL of standard or sample were injected.

2.3.8 Flow cytometry

For flow cytometry cell samples were treated according to Akerlund et al. (1995) by fixation in 70 % ethanol. Prior to flow cytometry, samples were centrifuged and resolved in buffer (10 mM Tris, 10 mM $MgCl_2$, pH 7.4). After recentrifugation and resuspension of the pellet in Tris-$MgCl_2$-buffer cells were stained with ethidium bromide (40 µg mL^{-1}) and mithramycin A (200 µg mL^{-1}). Flow cytometry was performed on a FACS Calibur (Beckton Dickinson). Small-angle forward light scatter was used for determination of cell size. The signal was calibrated with beads of 0.5 and 2 µm.

2.3.9 Rate determination of replication, transcription, and translation[§]

The rates of replication, transcription, and translation were estimated by incorporation of radioactive [Methyl-^{3}H]-thymidine, [^{3}H]-uridine and [U-^{14}C]-L-leucine (ICN Biomedicals). Therefore, 100 µL samples from shake flask or fermenter cultures of *E. coli* RB791 pKK177glucC were incubated at 37 °C for five minutes with 2 µCi [Methyl-^{3}H]-thymidine, 2 µCi [^{3}H]-uridine or 0.2 µCi

[§] This rate determination was performed by Dr. Peter Neubauer.

[U-^{14}C]-L-leucine. From the incubation vials 50 µL samples were added onto glass fiber filters (GF52, ∅ 2.5 cm, Schleicher and Schuell, Germany) in parallels. The filters were immediately placed in 10 % ice-cold perchloric acid for 30 min to stop the reaction. The filters were further washed twice with 5 % perchloric acid and twice with 96 % ethanol. After drying at room temperature the filters were put into tubes which were filled with 4 mL of Ultima gold XR Scintillator and measured in the Liquid Scintillation Analyser Tri-Carb 2100TR (Packard Instruments, U.S.A.).

2.3.10 Transmission electron microscopy of cell samples**

Fixation of the samples was performed by the Ryter and Kellenberger procedure (Ryter et al., 1958; Kellenberger & Ryter, 1964). The cells were prefixed directly in the culture medium with osmium tetroxide (final concentration 0.1 %, 30 min, 20 °C), washed after centrifugation (20 000 xg, 5 min) in Michaelis veronal-acetate buffer (MB, pH 6.2), which contained 0.25 g tryptone, 13 mL H_2O, 5mL stock solution (g 100mL^{-1}: 1.943 Na-acetate, 2.943 Na-veronal, 3.400 NaCl), 0.25 mL 1M $CaCl_2$, 7 mL 0.1 N HCl. After recentrifugation the pellet was resuspended in MB buffer containing an equal volume of osmium tertoxid (2 % w/v) and incubated overnight. After washing three times in MB the cells were included into low-gelling agarose (Merck, 2 % in MB), and small agar blocks of 1 mm^3 were cut. The specimen were washed again three times in MB, postfixed with uranyl acetate (0.5 % in MB, 2 h), dehydrated with a graded series of ethanol, transferred stepwise into propylene oxide and gradually over three steps (propylene oxide : resin = 3:1, 1:1, 1:3, 1 h each) into glycidether 100 × (Roth, Germany). Ultrathin sections were cut on an Ultracut S ultramicrotome (Leica, Vienna/Austria) using a diamond knife, stained with 4 % uranyl acetate and with lead citrate according to Reynolds (1963) and examined with a transmission electron microscope Zeiss EM 906 (Jena, Germany) at 60 kV.

2.4 On-line measurements and calculation

2.4.1 On-line measurements

Oxygen and carbon dioxide in the exhaust air were respectively measured by a paramagnetic and an infrared analysator with an Uras 10E (Hartmann & Braun, Germany). The dissolved oxygen tension (DOT) was continuously measured with a polarographic oxygen electrode (Ingold, Switzerland).

**This analysis was performed by Dr. Hanschke at University of Greifswald.

2.4.2 Kinetic parameters

The specific growth rate (μ), specific substrate consumption rate (q_s) and specific product formation rate (q_p) in the batch-phase were calculated according to the following equations:

$$\mu = (dX / dt) / X \quad (16)$$

$$q_s = (dS / dt) / X \quad (17)$$

$$q_p = (dP / dt) / X \quad (18)$$

where	μ:	specific growth rate	[h^{-1}]
	X:	dry cell weight	[g L^{-1}]
	t:	cultivation time	[h]
	q_s:	specific substrate consumption rate	[g $g^{-1}h^{-1}$]
	S:	substrate concentration	[g L^{-1}]
	q_p:	specific product formation rate	[g $g^{-1}h^{-1}$]
	P:	product concentration	[g L^{-1}]

2.4.3 Glucose uptake capacity

The maximal glucose uptake capacity ($q_{glucose\text{-}max}$) was calculated from the change of glucose to biomass concentration in growth phases where the glucose concentration was saturating the uptake systems. In contrast to this, pulses of glucose (0.5 to 1g L^{-1} final concentration) were added to the cultivations in the glucose limited growth phase. $q_{glu\text{-}max}$ was calculated from analyzed samples which were frequently collected after the pulse as well as from the DOT curve after the pulse if the respiration was not limiting as described in the results section 3.2.6. The medium samples were directly sucked from the reactor through a 0.2 μm disc filter into a syringe.

2.4.4 Respiration data

The specific rates (q_{O2}, q_{CO2}) were calculated continuously over the fermentation by including the fitted biomass values and the calculation of the actual volume on the total reactor weight corrected for the broth density.

$$OUR\ [mmol\ L^{-1}h^{-1}] = \frac{Q}{V} \times \frac{273}{(T+273)} \times \frac{(C_{O2in} - C_{O2out}) / 100}{R} \quad (19)$$

$$q_{O2}\ [mmol\ g^{-1}h^{-1}] = OUR / X \quad (20)$$

$$CER\ [mmol\ L^{-1}h^{-1}] = \frac{Q}{V} \times \frac{273}{(T+273)} \times \frac{(C_{CO2out} - C_{CO2in}) / 100}{R} \quad (21)$$

$$q_{CO2}\ [mmol\ g^{-1}h^{-1}] = CER / X \quad (22)$$

$$RQ = CER / OUR \quad (23)$$

where:	OUR:	volumetric oxygen uptake rate	[mmol L^{-1} h^{-1}]
	Q:	outlet gas flow rate	[L h^{-1}]
	V:	culture volume	[L]
	T:	temperature	[°C]
	$C_{O2in:}$	oxygen concentration in inlet gas	[%]
	$C_{O2out:}$	oxygen concentration in outlet gas	[%]
	R:	standard gas constant	= 22.4 [L mol^{-1}]
	q_{O2}:	specific oxygen uptake rate	[mmol $g^{-1}h^{-1}$]
	CER:	volumetric carbon dioxide evaluation rate	[mmol $L^{-1}h^{-1}$]
	$C_{CO2out:}$	carbon dioxide concentration in inlet gas	[%]
	$C_{CO2in:}$	carbon dioxide concentration in outlet gas	[%]
	$q_{CO2:}$	specific carbon dioxide evaluation rate	[mmol $g^{-1}h^{-1}$]
	RQ:	respiratory quotient	[mol CO_2 mol^{-1} O_2]

3 Results

In this thesis, a yeast α-glucosidase produced in *E. coli* was used as model system in order to obtain a more comprehensive knowledge about cell physiology in response to overexpression of heterologous genes during glucose limited fed-batch cultures. A limited number of studies was also performed with two other heterologous products, CRIMI (creatinine imino hydrolase) and ZZ protein, which were investigated in frame of an industry cooperation and a European network project. The combination of the experiences obtained from these different systems and the evaluation of the current literature on this subject, allows to make some broader conclusions about the cell behavior under strong recombinant gene induction. However, I specifically want to adress that the aim of this work was not to indicate the whole range of validity of the findings for the huge number of expressed proteins, but to describe thoroughly one model system. On this basis ongoing studies in our laboratory and of other groups will show which effect is caused by the specific properties of the protein and/or the expression system.

The experiments were generally performed under process-like conditions, namely by induction during the glucose limited growth phase of a fed-batch fermentation. Whereas the study is focusing in the first part on cellular responses and the maintenance of cell viability, certain specific questions in connection to the production scale are investigated in the second part of this work.

3.1 Cell growth and segregation in recombinant bioprocesses

3.1.1 Cell growth in recombinant *E. coli* fed-batch cultivations

In most cases, the overproduction of recombinant proteins to a high level of the cell protein results in an inhibition of the cellular growth. Although the inhibition of growth is also obvious in shake flask cultures, I used a simple glucose limited fed-batch fermentation process with a constant feed rate, which is a common method to reach higher cell densities and therefore to increase volumetric yields. Although, in many industrial processes an exponential feed is often used after an initial batch phase for a certain period, I used the constant feed method due to its higher reproducibility, and as the inducing signal is mostly given to the culture in industry when the feed was switched to a constant rate.

Growth inhibition after induction of recombinant α-glucosidase. Fed-batch cultivations for α-glucosidase production were performed with the recombinant strain *E. coli* RB791 containing the plasmid pKK177glucC. This host-vector system was partially used in connection with the pUBS520

plasmid for coexpression of the rare arginyl tRNA (argU). As the gene for α-glucosidase contains 19 aga/agg codons, corresponding to 3.23 % of the total α-glucosidase codon usage, which is ten times more than the average use of these codons in *E. coli*, the formation of the recombinant product is limited by argU tRNA as shown by Brinkmann et al. (1989). An approximately fourfold increase of the cellular product concentration was obtained by cotransformation of the pUBS520 plasmid (Fig. 3.1b,f).

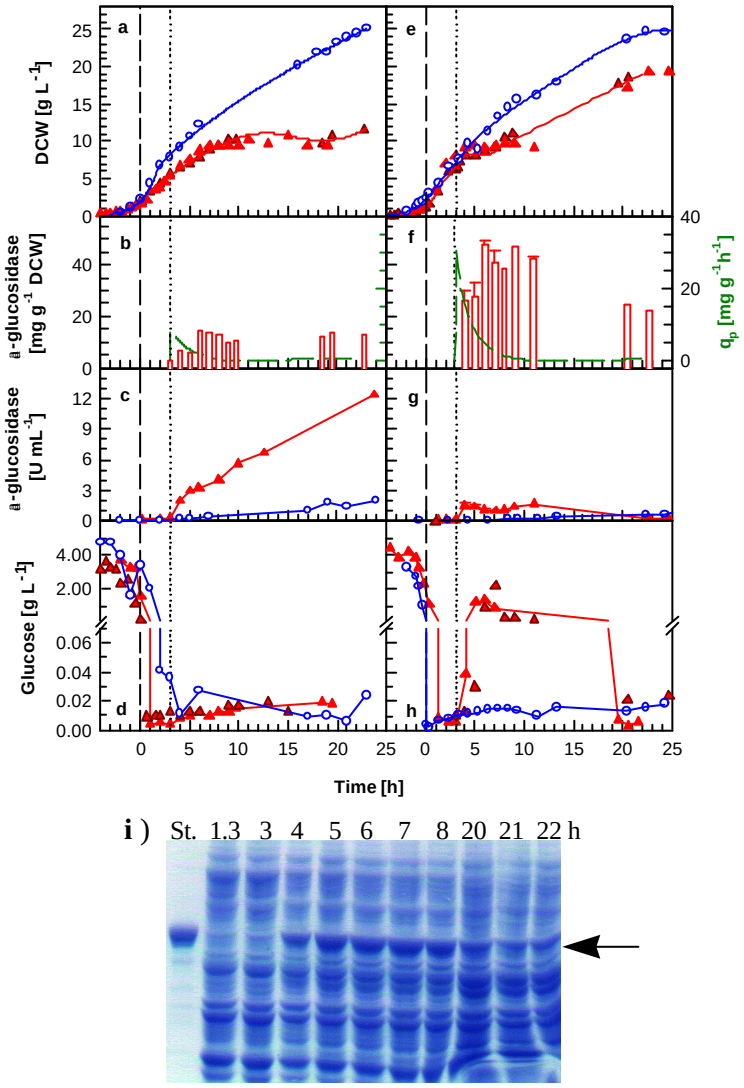


Figure 3.1: Growth characteristics of glucose limited fed-batch fermentations with *E. coli* RB791 pKK177glucC (**a-d**) and *E. coli* RB791 pKK177glucC pUBS520 (**e-h**) in dependence on the addition of IPTG for induction of the α-glucosidase (open symbol: no induction, filled symbol: induction by addition of IPTG). (**a**, **e**) biomass; (**b**, **f**) α-glucosidase concentration per dry cell weight after induction and specific product formation rate (---); (**c**, **g**) activity of soluble α-glucosidase; and (**d**, **h**) glucose concentration. The interrupted line represents the time of feeding start at a cell density of approximately 2 g L^{-1}. The dotted line indicates the addition of IPTG (1 mM final concentration). (**i**) SDS-PAGE analysis of glucose limited fed-batch fermentations of *E. coli* RB791 pKK177glucC pUBS520 with induction of 1 mM IPTG. Zero time indicates the point of feeding start.

Glucose limited fed-batch fermentations with a constant feed were performed with and without addition of IPTG as the inducing agent of the recombinant α-glucosidase. During the batch phase with 5 g L^{-1} glucose the strains grew with a maximum growth rate according to OD_{500} of 0.68 ± 0.08 h^{-1} (without pUBS520) and 0.79 ± 0.05 h^{1} (with pUBS520). Glucose feeding was started at a defined cell density at the end of the batch phase causing a further exponential growth of the cells to the point of glucose exhaustion. From the point of glucose limitation the glucose concentration was

detected to be below 15 mg L^{-1} according to the low K_s value for glucose of *E. coli* and stayed at this low level up to the end of those cultivations without induction or with induction but without pUBS520. In the culture with pUBS520, the glucose concentration increased to about 1.5 g L^{-1} after addition of IPTG possibly due to a decrease of the glucose uptake capacity of the cells following induction. The specific growth rate μ decreased continuously due to the constant feed rate and the cell dry weight steadily increased to a value of about 25 g L^{-1}. Acetate accumulated during the batch phase to a maximum concentration of about 0.6 g L^{-1} and was reconsumed within three hours after the onset of glucose limitation. α-glucosidase was very low in the fermentations without induction and could only be detected by activity analysis for the soluble protein (Fig. 3.1c,g).

When the inductor IPTG was added three hours after feeding start, α-glucosidase formation proceeded at a higher rate for approximately 3 hours (Fig. 3.1b,f), whereby the specific rate of α-glucosidase formation was much higher in the strain containing the pUBS520-plasmid than in the strain without the plasmid. In both systems, the specific product formation stops about 3 hours after induction which is connected to growth inhibition in the strain RB791 pKK177glucC pUBS520 (Fig. 3.1e), but not in the strain without pUBS520, in which growth was blocked only from seven hours after induction (Fig. 3.1a). The product appears to a dominant part in inclusion bodies and is about 4 % of the total cellular protein in the strain without pUBS520 but about 16 % of the total cellular protein in the strain containing pUBS520. In the case of these cultivations only less than 5 % of the product was soluble and showed activity, whereby the activity was lower in the system with coexpression of the argU tRNA (Fig. 3.1c,g). In principle, the soluble part of the α-glucosidase could be increased by changing cultivation conditions such as pH and temperature, as well as by lowering the amount of IPTG or induction by lactose in a *lacY* mutant (Kopetzki et al., 1989a,b). Although optimization of the α-glucosidase activity was not the aim of this thesis, the activity (Fig. 3.1c,g) was measured and found that it increased after induction.

Effect of CRIMI production on cell growth. The plasmid pDSCrimi for overproduction of CRIMI was transformed into the same host strain *E. coli* RB791 and the cultivation was exactly performed by the same scheme as for α-glucosidase production in order to compare the two systems. Time profile of cell growth, product formation, substrate consumption and the corresponding specific rates are presented in Fig. 3.2. During the initial batch phase the cells grew exponentially with a maximum specific growth rate μ_{max} of 0.80 h^{-1}. The maximum specific glucose uptake rate q_{smax} in the batch phase was determined to be 1.2 g g^{-1} h^{-1}. The biomass yield on glucose

was 0.64 g g^{-1}. From the point of glucose limitation the glucose concentration was detected to be below 40 mg L^{-1} and stayed at this low level up to the end of the cultivation. In contrast to the α-glucosidase process, the growth proceeded also after induction of the CRIMI and the final cell dry weight was 22 g L^{-1}.

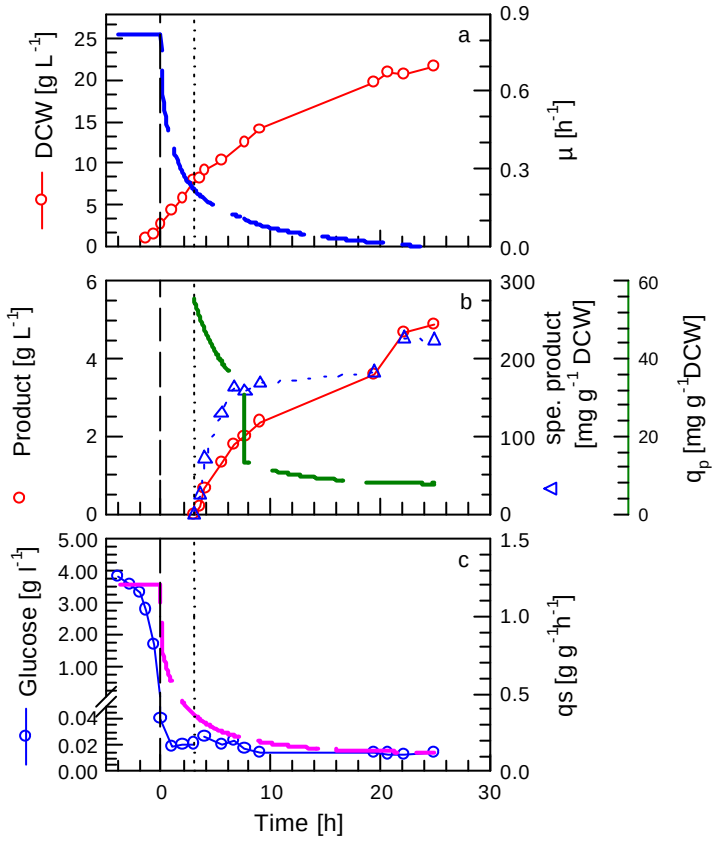


Figure 3.2: Growth characteristics of glucose limited fed-batch fermentation with *E. coli* RB791 pDSCrimi. (**a**) cell dry weight (○) and specific growth rate (μ,----), (**b**) creatinine imino hydrolase concentration (○-g L^{-1}, Δ-mg g^{-1} DCW) and specific product formation rate (q_p,---), (**c**) glucose concentration (○) and glucose uptake rate (q_s, ----). The interrupted line represents the time of feeding start at an cell density of approximately 2 g L^{-1}. The dotted line indicates the addition of IPTG (1mM final concentration).

After induction the concentration of CRIMI increased with a rate of 45 mg g^{-1} h^{-1} and reached to a level of 160 mg g^{-1} DCW about four hours after induction, corresponding to about 30 % of the total cell protein. The total concentration of CRIMI at this time was about 1.7 g L^{-1} (Fig. 3.2c). During further cultivation the product concentration increased approximately linearly to about 5 g L^{-1} to the end of the cultivation. About two third of the product accumulated in the insoluble fraction and one third was soluble and active.

In difference to the strong inhibition effect of α-glucosidase production (Table 3.1), the synthesis of CRIMI had only minor effect on the growth. The inhibition of cellular growth following induction in the α-glucosidase system was further investigated by analyzing the cell populations to see what kind of cell segregation occurs following induction.

Table 3.1: Comparison of cultivation parameters of glucose limited fed-batch processes for production of α-glucosidase and CRIMI in *E. coli* RB791.

	RB791 pKK177glucC	RB791 pKK177glucC pUBS520	RB791 pDSCrimi
μ_{max} [h^{-1}]	0.68	0.79	0.82
qs_{max} [g g^{-1} h^{-1}]	1.1	1.34	1.21
$Y_{x/glucose}$	0.29	0.46	0.64
q_p [mg g^{-1} h^{-1}]	3.2 (initial q_p)	15.5 (initial q_p)	45
P [mg g^{-1}]	15	50	160
Ratio of IB/soluble	99:1	99:1	2:1
DCW/OD_{500}	0.22 [a] / 0.27 [b]	0.26 [a] / 0.21 [b]	0.29 [a] / 0.18 [b]
$k_{d\ cfu}$ [h^{-1}]	0.35	0.74 [c] / 0.27 [c]	0.10

[a]: before induction, [b]: after induction, [c]: two phase process, more details see Fig.3.3.

3.1.2 Cell segregation and plasmid stability after IPTG induction

By replica plating it was shown that the growth inhibition after induction of α-glucosidase production is connected to a strong decrease in the ability of the cells to form colonies on agar plates. In the case of *E. coli* RB791 pKK177glucC, the ability to form colonies decreased by a specific rate constant of 0.35 h^{-1} from 100 % at 3 h after induction to about 1 % within 15 hours (Fig. 3.3b). In contrast to the control culture where no significant cell segregation was found, more than 80 % of the culture reached the state of non-dividing cells within a few hours, shortly after accumulation of α-glucosidase.

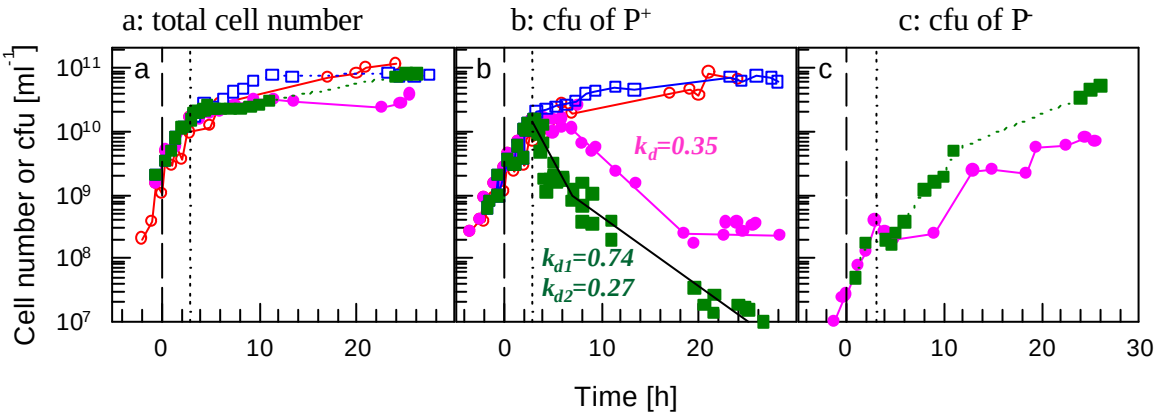


Figure 3.3: Comparison of (**a**) total cell number, (**b**) colony forming units of plasmid-containing cells (P^+) and (**c**) cfu of plasmid-free cells (P^-) during fed-batch cultivations of *E. coli* RB791 pKK177glucC (○, ●) and *E. coli* RB791 pKK177glucC pUBS520 (□, ■) in dependence on the addition of IPTG for induction of the α-glucosidase (open symbol: no induction; filled symbol: induction by addition of 1 mM IPTG).

The analysis of glucose (see Fig. 3.1d) showed that all the carbon which was fed continuously to the culture was taken up in the system without pUBS520, which proved that the culture, although non-growing, exerted metabolic activity. This was further supported by analysis of the respiratory activity. The specific consumption rates of oxygen (q_{O2}) and carbon dioxide (q_{CO2}) were not significantly

lower than the rates of a non-induced culture (Fig. 3.4). A fluorescence assay for characterization of living from dead cells (BacLight, Molecular Probes, USA) based on the different permeability of the membrane for the two fluorescence markers showed that all cells were viable.

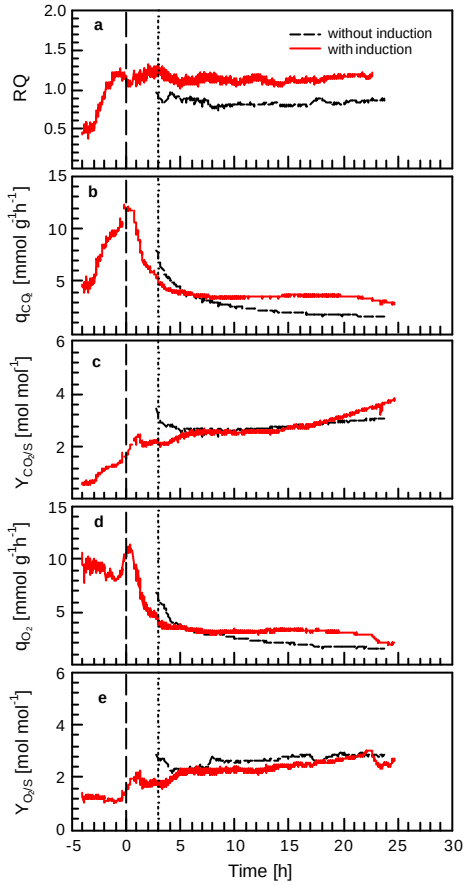


Figure 3.4: Fed-batch cultures of *E. coli* RB791 pKK177glucC without induction (--) or with induction (—) by 1 mM IPTG. Comparison of the respiratory quotient (RQ, **a**), carbon dioxide evaluation rate (q_{CO2}, **b**), oxygen uptake rate (q_{O2}, **d**), yield of carbon dioxide and oxygen per glucose ($Y_{CO2/S}$, **c**; $Y_{O2/S}$, **e**).

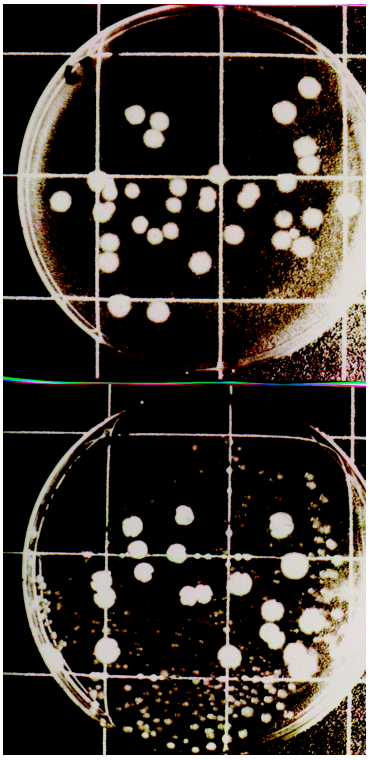


Figure 3.5: Colonies grown on plates: (**a**) before induction all colonies were of the same size; (**b**) 10 h after induction culture segregation into small and large colonies.

The recovery of the cells on both, NBII plates with and without the antibiotic ampicilline, was not abruptly inhibited, but the cells recovered on the plates with a different time delay. In difference to a non-induced cell which formed large colonies within 10 hours, after induction the small portion of cells which was able to form colonies, showed a time delay of up to 48 hours (Fig. 3.5). These smaller colonies were counted as positive according to their colony forming ability. However, more than 80 % of the cells from 6 h after induction did not recover to growth. Concluding from these results, the population of cells which is shifting to the non-growing state is viable but incompetent for further division.

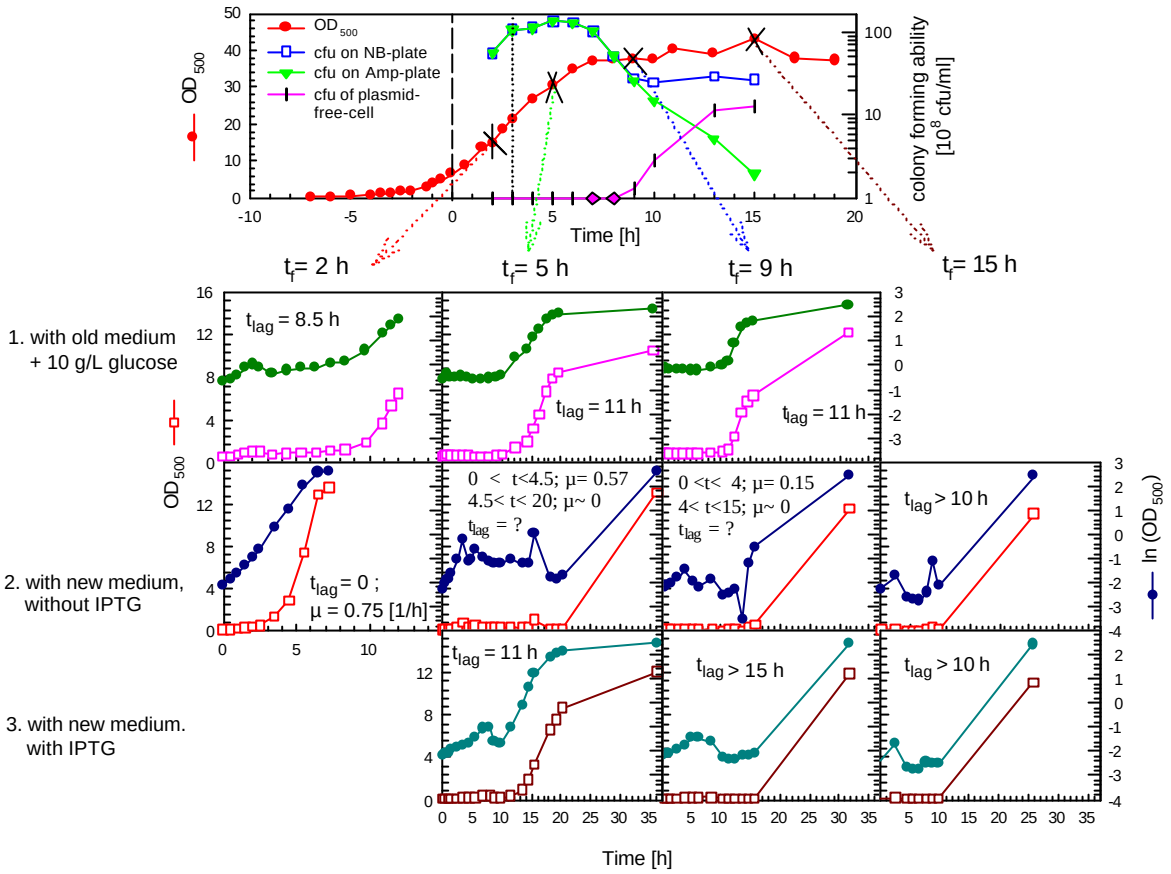


Figure 3.6: Reinoculation experiments into shake flasks with new mineral salt medium with cells from different phases during the fed-batch fermentation of *E. coli* RB791 pKK177glucC.

To exclude that an inhibitory compound in the medium is causing growth inhibition in the case of induced cells, samples were inoculated to fresh or spent medium where only new glucose was added. In both cases, samples from the fermentation with a large dividing-competent population started to growth nearly immediately (Fig. 3.6). However, a long lag phase was observed in fresh as well in spent medium, if cells were taken as inocolum from the phase of the fermentation where more than 90 % of the cells were unable to recover to growth on plates. The growth of these cultures was contributed to the overgrow of part of the cells which were also found to grow on plates.

The growth inhibition was stronger if the strain with the plasmid pUBS520 was used. In this system, growth inhibition started earlier and the colony forming ability decreased by a higher specific rate of 0.74 h^{-1} during the first 3 hours after induction. After this first period the k_d decreased to 0.27 h^{-1}. Furthermore, as obvious in Fig. 3.3c, in all cultures performed for α-glucosidase production, cells which had lost the ampicilline resistance could be detected by replica plating. The number of these cells, which obviously do not contain the pKK177glucC plasmid, increased to the end of the fermentations. This cell population contributed to about one third of the total cell number 20 hours

after IPTG addition in *E. coli* RB791 pKK177glucC pUBS520. No plasmid loss was observed in the control cultivations without induction. In contrast to the plasmid pKK177glucC, the second plasmid pUBS520 with the kanamycine resistance was always stabily maintained.

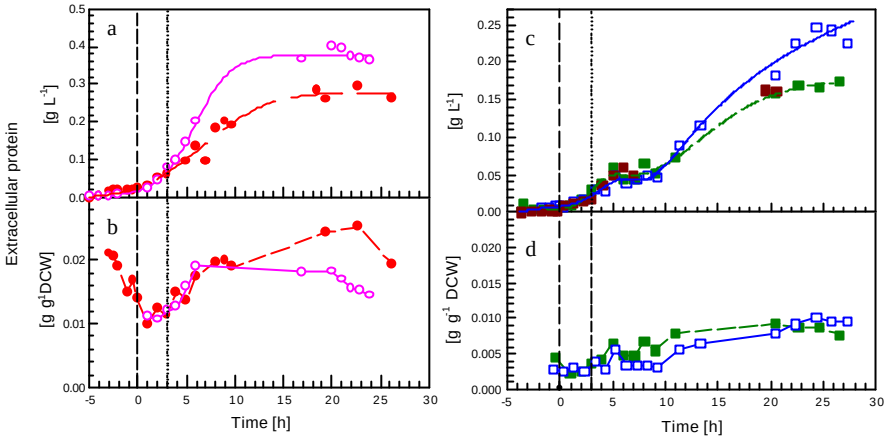


Figure 3.7: Comparison of extracellular protein content during fed-batch fermentations of *E. coli* RB791 pKK177glucC (○, ●) and *E. coli* RB791 pKK177glucC pUBS520 (□, ■) in dependence on induction of the α-glucosidase (open symbol: no induction, filled symbol: induction by addition of IPTG).

Extracellular protein was measured to investigate to which amount the non-dividing population is contributing to cell lysis. The specific extracellular protein content reached up to a level of 0.02 g g^{-1}DCW for the cells with pUBS520 and 0.008 g g^{-1}DCW for the cells without pUBS520, which indicated that the cells without pUBS520 had a higher lysis rate (Fig. 3.7b,d). Although the cell growth was inhibited by the induction of α-glucosidase, in connection to cell lysis there was no significant difference between the cultures with and without induction. These results suggest that the non-dividing cells were not lysing. The onset of increasing accumulation rate of extracellular proteins correlated with the time when cultivation was shifted from batch phase to limited growth in both systems. Surprisingly, in the culture with pUBS520 the cells lysis was also inhibited after induction for about 4 hours.

Effect of CRIMI production on cell segregation and plasmid stability. The total cell number and colony forming units during cultivation of *E. coli* RB791 pDSCrimi are represented in Fig. 3.8. No up-growth of plasmid-free cells was observed after induction in this strain, but a part of the cell population lost the ability to divide. The rate of the development of a non-dividing population was detected to be about 0.10 h^{-1} from 3 hours after induction. Under this constant rate the part of the population, which has lost its colony forming ability, was about 90 % of the total cells at the end of

the experiment. The analysis of extracellular protein indicated that the lysis rate is higher direct after induction with a maximum level of 1.7 mg $g^{-1}h^{-1}$.

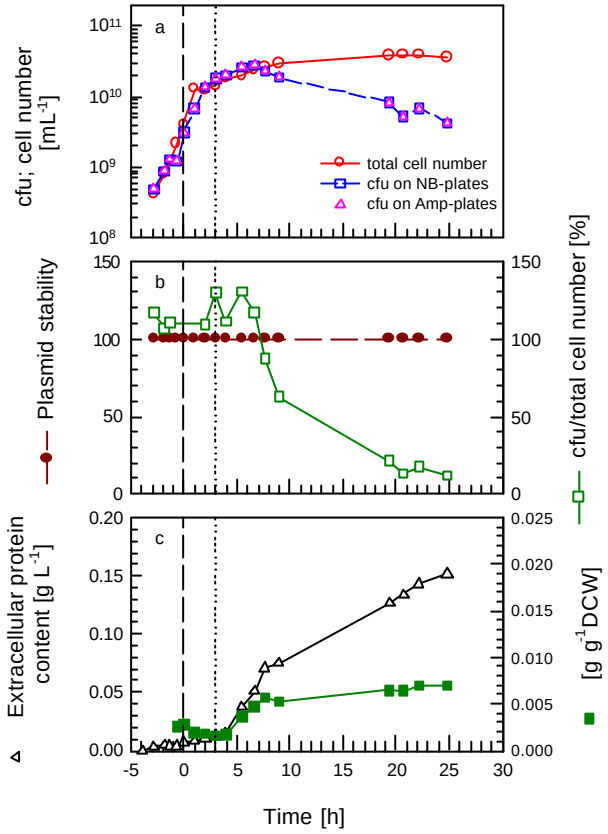


Figure 3.8: Cell segregation and plasmid stability of fed-batch fermentation with *E. coli* RB791 pDSCrimi. a) Total cell number (●) and colony forming units (cfu; which are presented as total cfu on NB plates (□) and the part of ampicilline resistant cells (Δ)), b) Plasmid stability (●) and cell segregation into viable but no culturable cell (□), c) extracellular protein content (g L^{-1}, Δ; g g^{-1}DCW, ■). Induction was performed by addition of IPTG to a final concentration of 1mM (dotted line).

In summary, significant differences were detected in connection to the influence of the recombinant production on the cellular growth, cell survival and plasmid stability, between the two recombinant processes, although both products, α-glucosidase and CRIMI, accumulated to more than 10 % of total protein. Furthermore, in both systems the cell segregation into non-dividing cells was observed and the population of cells which is shifting to the non-growing state is viable but incompetent for further division. This phenomenon was earlier described by Andersson et al. (1996a,b). The authors showed that the non-dividing cell population has certain metabolic activity, such as respiration and glucose uptake, and was therefore discussed to fulfill the requirements of the viable but non-culturable (VBNC) status, which is known from the environmental microbiology. However, the VBNC status in recombinant cultures is neither well documented, nor really proved. From the available data it is not clear, why this segregation occurs and what is the molecular background of the growth inhibition. Therefore, in the following chapter the status of the cells will be more thoroughly described by investigating general cellular processes and the energetic situation of the cell in connection to induction of the recombinant product in glucose limited fed-batch cultivations.

3.2 Cellular responses after strong induction of recombinant α-glucosidase

3.2.1 Activity of replication, transcription and translation

Inhibition of cellular reactions as a consequence of α-glucosidase formation. The growth inhibition in α-glucosidase expression systems, with and without pUBS520, is connected to the inhibition of cellular reactions, such as replication, transcription, and translation. The activity of replication, transcription, and translation were investigated by radioactive incorporation experiments. Fig. 3.9 shows by incorporation of [^{14}C]-L-leucine and [^{3}H]-thymidine that translation and replication are inhibited to more than 80 % within three hours (translation) or two hours (replication) respectively in a shake flask culture of *E. coli* RB791 pKK177glucC. In contrast to this, transcription was rather stable. There was a reduction of [^{3}H]-uridine incorporation to 50 % within approximately 4 h after induction.

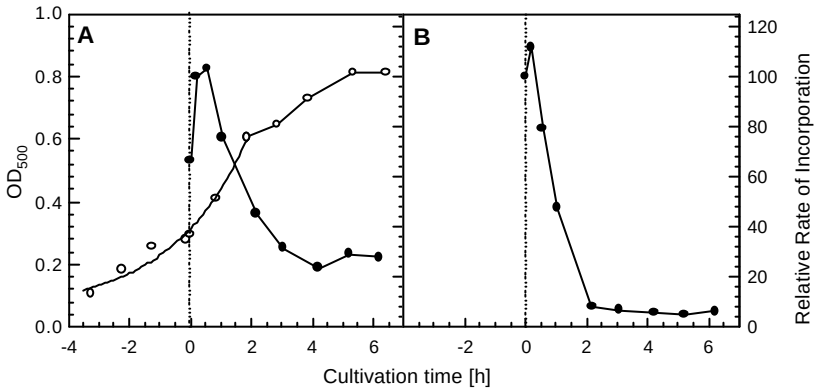


Figure 3.9: Inhibition of translation (**a**) and replication (**b**) (–●–), measured by the relative incorporation rate of [^{14}C]-leucine or [^{3}H]-thymidine respectively following induction of α-glucosidase gene expression in shake flask cultures of *E. coli* RB791 pKK177glucC. The incorporation of the radioactive molecules was calculated on a cell basis and was set to 100 % at the time of induction. Growth of the culture is shown by OD_{500} (–○–). Induction was performed by addition of 1 mM IPTG at time zero.

However, all three reactions were very fast inhibited in the cells containing the pUBS520 plasmid which are suspected to contain a higher content of the *arg*U tRNA. This was shown not only in shake flask cultures (data not shown), but also in a glucose limited fed-batch fermentation (Fig. 3.10). In the fermentation, all three processes, replication, transcription, and translation were inhibited much faster than in a control fermentation without induction, where these processes were very much related to the decrease of the specific growth rate. The translational activity was decreased to about 50 % during the glucose limited growth (Fig. 3.10), whereas the transcription was barely influenced by the growth rate reduction. After induction of α-glucosidase production the incorporation of [^{14}C]-L-leucine was further reduced to about 10 % of the rate which was measured

during logarithmic growth. A slight increase of the rate was measured from 5 hours after induction, which is related to the upcoming plasmid-free cell population. The level of transcription was even more reduced after IPTG addition to about 3 % of the level before induction, but recovered to about 30 % if the plasmid-free cells were growing up. The incorporation of [^{3}H]-thymidine was even faster inhibited than translation, which again shows the high dependence of replication not only on translation, but on the synthesis of a limiting factor which has recently discussed as the DnaA protein (Kogoma, 1997).

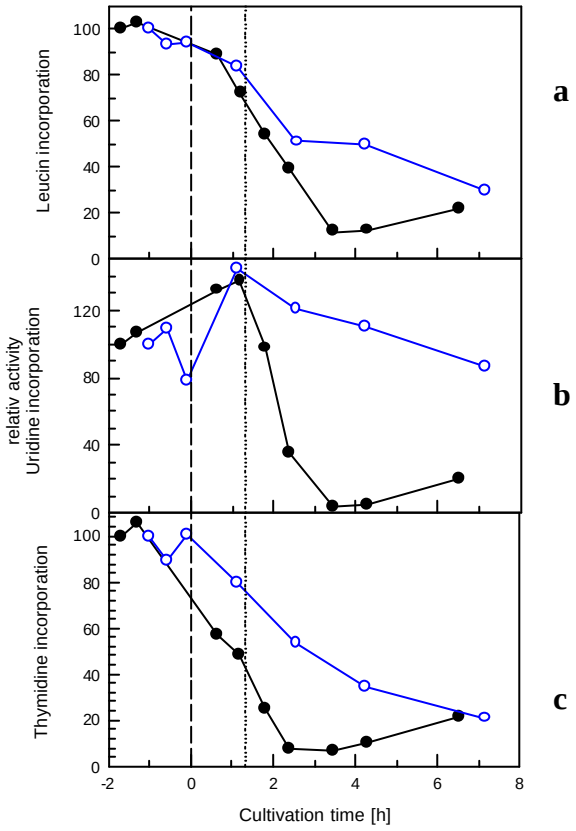


Figure 3.10: Comparison of the relative incorporation rate of [^{14}C]-L-leucine (**a**), [^{3}H]-uridine (**b**), and [^{3}H]-thymidine (**c**) as a measure of translation, transcription and replication activity in fed-batch cultivations of *E. coli* RB791 pKK177glucC pUBS520 without induction (as control, open circles) and with induction by 1 mM IPTG (closed circles). The incorporation of the radioactive molecules was calculated on a cell basis and the values from the batch phase of growth were set to 100 %. Induction was performed by addition of 1mM (final concentration) of IPTG (dotted line).

In summary, it has been shown by the incorporation experiments that the overexpression of α-glucosidase strongly influenced the activity of replication, transcription and translation, but to a different level. Transcription was most strongly inhibited, what would have consequences for the induction of stress responses after induction of the α-glucosidase. I assume that this inhibition is a direct maintenance effect, that means an effect of the competition of the large amount of recombinant mRNA for the available ribosomes and therefore a reduction of important proteins which are involved in these processes.

3.2.2 Plasmid amplification after induction

In order to better understand the appearance of plasmid-free cells following induction, namely, whether plasmids are quickly degraded after induction by a process which could be similar to that, published by Neubauer et al. (1996), or whether this plasmid-free population is caused by an up-growth of plasmid-free cells by the growth advantage which these cells would have, if the growth of the producing cells is inhibited, we followed the plasmid content in glucose limited fed-batch cultivations of *E. coli* RB791 pKK177glucC before and after induction. Although there were no indications in the literature for a changing plasmid copy number after induction of P_{tac} promoter controlled expression systems, the plasmid content increased on a cellular basis from about 100 copies per cell to about 400 copies after induction corresponding to a factor of four (Fig. 3.11). In contrast to this observation, no increase of the plasmid copy number per cell was found in cultivations without induction. The increase of the plasmid content was partly connected to the accumulation of multimers of the plasmid pKK177glucC, which were visible on agarose gels (Fig. 3.12b).

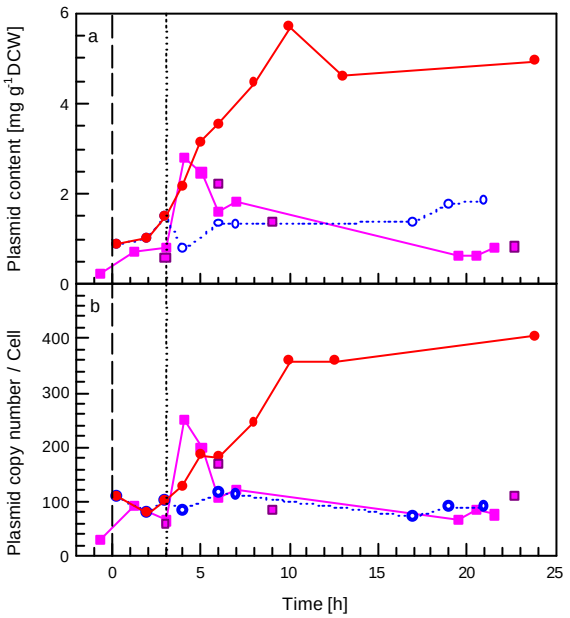


Figure 3.11: Comparison of plasmid content (**a**) and plasmid copy number based on plasmid-containing cells (**b**) during fed-batch fermentations of *E. coli* RB791 pKK177glucC (○, ●) and *E coli* RB791 pKK177glucC pUBS520 (■) in dependence on the addition of IPTG for induction of the α-glucosidase (open symbol: no induction, filled symbol: induction by addition of IPTG). The feeding started at zero hours (interrupted line) before glucose was exhausted. Plasmid concentrations were calculated from spectrophotometrical analysis and were average values of at least three independent preparations.

Based on the replication rate which decreased to approximately 8 % of the value measured before IPTG addition, within two hours after induction (Fig. 3.10b) by measuring the incorporation of [^{3}H]-

thymidine, the relative incorporation rate of 8 % corresponds fairly well to the calculated plasmid DNA content of an *E. coli* cell (about 100 copies of the plasmid pKK177glucC with a size of 4.675 kbp), which is less than 8 % of the total DNA concentration of the cell (on assumption of a chromosome number per cell of 1.85, chromosome size of 4700 kbp, calculated from data given by Bremer and Dennis, 1996). From these results, we conclude that chromosomal replication is nearly completely inhibited within two hours after induction. Furthermore, it is obvious from Fig. 3.10b that stable replication (which includes plasmid replication) approximately proceeds with a constant incorporation rate. Therefore, we assume that replication proceeds only with the number of plasmids which were replicating before amplification started. There seems to be no new formation of replication complexes, and therefore the plasmid copy number increases linearly, confirming the measurements of Fig. 3.11b.

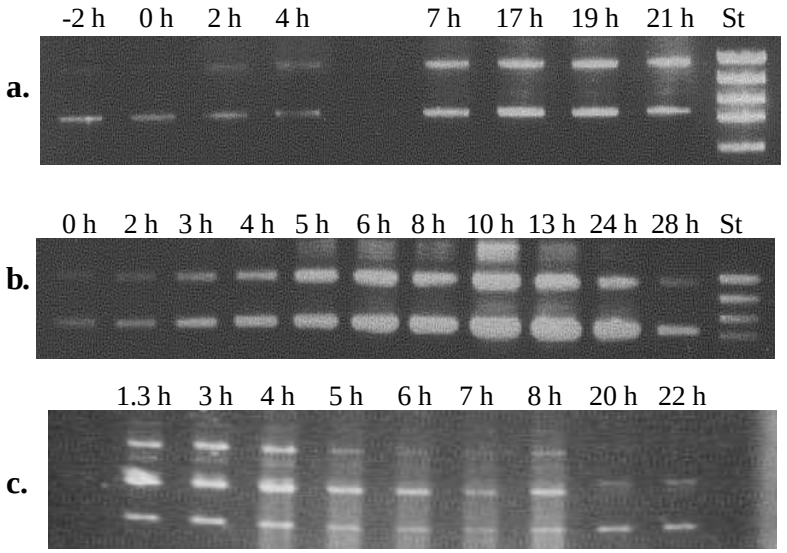


Figure 3.12: Plasmid preparation of samples from the fed-batch cultivations of *E. coli* RB791 pKK177glucC without induction (**a**) and with induction (**b**) and of *E coli* RB791 pKK177glucC pUBS520 with induction (**c**). The samples were extracted from the same amount of cells according to OD_{500} value by the Qiagen method. The feeding was started at zero hours. The decrease of the plasmid content in this sample is due to the up-growth of plasmid-free cells. The different DNA bands are different isomers of the pKK177glucC plasmid, which can be transferred to the linear plasmid form by HindIII digestion (St - DNA size standard, 1 kb ladder).

A transient plasmid amplification was also observed by a factor of two in the system containing the both pKK177glucC and the pUBS520 plasmids (Fig. 3.11). However, plasmid amplification was only observed for the pKK177glucC plasmid and not for pUBS520 which maintained at a level of 10 copies per cell.

In contrast to the pKK177glucC plasmid, the plasmid pDSCrimi was stabily maintained through the cultivation, and no significant amplification occurred after induction, just a slight increase of the plasmid copy number (Fig. 3.13).

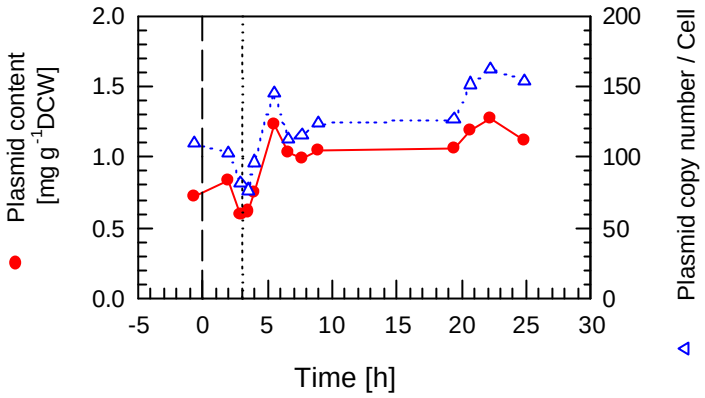


Figure 3.13: Plasmid content (●) and plasmid copy number (Δ) based on plasmid-containing cells during fed-batch fermentations of *E. coli* RB791 pDSCrimi with induction by addition of IPTG (dotted line). The feeding started at zero hours (dashed line) before glucose was exhausted.

Furthermore, ColE1-derived plasmids containing different recombinant genes which are controlled by the *tac* promoter were amplified following induction with IPTG, but no amplification occurred if product formation was not induced. Plasmid amplification occurred in *E. coli* B strains as well as in K-12 strains with different plasmids (*rop*$^+$ and *rop*$^-$) coding for various heterologous proteins. The amplification was not caused by a toxic effect of IPTG, but was related to a strong inhibition of translation and chromosomal replication after the induction of heterologous gene expression (for further results and discussion see Teich et al., 1998).

3.2.3 Influence of α-glucosidase production on the chromosomal DNA supercoiling

Inhibition of cellular reactions as a consequence of α-glucosidase formation and the plasmid amplification due to the inhibition of chromosomal replication were described in the sections above. Here I wanted to investigate the status of induced cells. Similar to Andersson et al. (1996b), high product formation was connected to appearance of non-culturable cells, which had not lost all metabolic activities, and even succeeded to maintain some glucose uptake and respiratory ability. The development of a non-dividing population with a certain metabolic activity after overexpression of a recombinant gene was sometimes compared to the status of viable but non-culturable cells (Andersson et al., 1996b). However, the results from overproduction of α-glucosidase might also imply that the non-dividing cells perform the intermediate state from growing to dead cell.

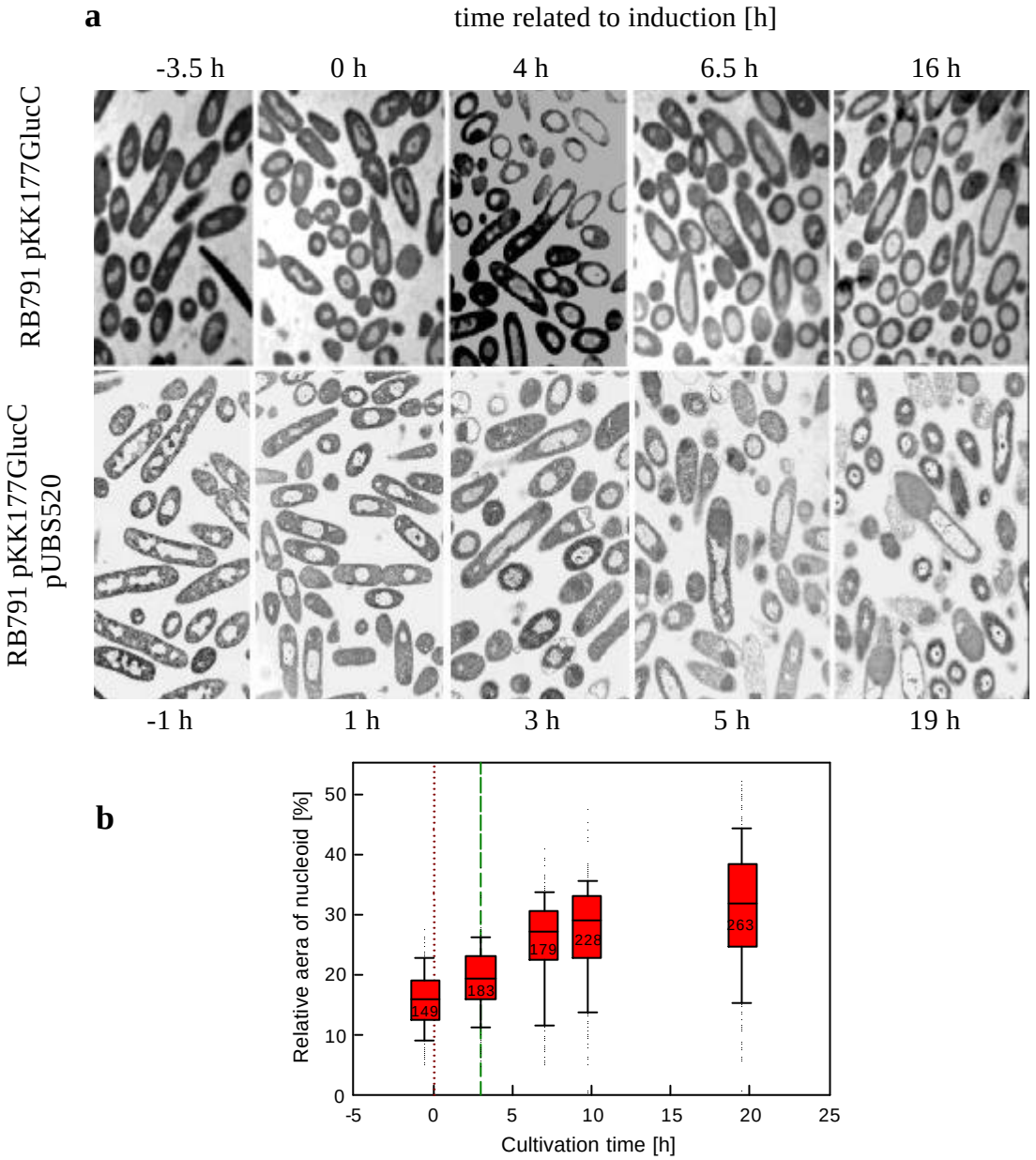


Figure 3.14: Electron microscopic images **(a)** of cells during different phases of glucose limited fed-batch fermentations of *E. coli* RB791 pKK177glucC (upper panel) and of *E. coli* RB791 pKK177glucC pUBS520 (lower panel) with induction of α-glucosidase gene expression. The white area inside the cells corresponds to the chromosomal DNA which becomes visible after uranyl acetate treatment. The average relative area of the nucleoid relative to the total cell area from the fermentation of *E. coli* RB791 pKK177glucC was calculated after treatment of the photographs with an image analysis procedure, which is described in the methods section and is presented in **(b)**. The numbers indicate the evaluated cells for each sample. The 25 % and 75 % quantils are shown by the grey boxes and bars respectively.

Among different possibilities which would make a cell unable to recover on plates, a damage of the chromosomal DNA would cause the cells to be unable to replicate if this damages would be not repaired. Because of the high synthesis of the recombinant product, which according to rough estimate from the specific product formation rate contributes to about 85 % of the total protein

synthesis shortly after induction and the strong decrease of translation and transcription shortly after induction, the induction of the SOS regulon, which is the cellular response to DNA damage, should be impaired after induction. However, single or double strand breaks in DNA which would cause an inability for replication, were not detected by electrophoretic methods or by using apoptosis test kits (data not shown). Also chloroquine incorporation experiments, which can be used to detect the degree of supercoiling of small plasmids, failed to detect the decrease of supercoiling of the pKK177glucC plasmid, as it is too large for these investigations.

However, there was indication in the literature (Hecker et al., 1986) that the cells, which are unable to induce the stringent response and therefore dying within some days if they are cultivated on pure phosphate buffer, show a relaxation of the chromosome. Therefore, electron microscopy studies were carried out for checking the change in the chromosomal DNA during the recombinant fermentations (Fig. 3.14). In electronmicroscopic images the chromosome of a vital cell appeared condensed and covered about 15 % of the cytoplasmic space (see Fig. 3.14). In contrast, the chromosome was extended to about 30 % of the cytoplasm in a non-dividing or dead cell. The electronmicroscopic analysis of samples from the fermentations with induction indicated a decrease in the packing density of the chromosome, which was obvious by the increase of the relative chromosomal area. This could be statistically proved by image analysis for *E. coli* RB791 pKK177glucC (Fig. 3.14b). The analysis was impossible with the high production strain with coexpression of the argU tRNA, as the large inclusion bodies disturb the image analysis. However, in this case a lot of cells, which had a decondensed chromosome, can be observed in the images.

From the results, the decrease in the density of the chromosome could be one important aspect of the loss of cell division ability. This could be caused in principal by different mechanisms which will be discussed in the following section.

3.2.4 Analysis of DNA binding protein (H-NS) and LexA protein after induction

As above mentioned, the loose of supercoiling could be caused by not repaired strand breaks, by the reduced synthesis of DNA binding proteins after induction, or by an uncontrolled abort of replication. High product synthesis might also influence the production of DNA binding proteins, because the recombinant product is synthesized in competition to the cellular proteins. Among them, H-NS seems to be the most important player connected to gene regulation. Spurio and coworkers (1992) showed that the adjustment of the H-NS concentration played an important role in cell

viability and nucleoid condensation. Therefore, the loss of DNA condensation could be assumed to be connected to a decrease in the H-NS concentration. In contrast to the expectation that the maintenance of H-NS was disturbed by the α-glucosidase production, the level of H-NS did not significantly decrease after induction of the α-glucosidase within four hours after induction (see Fig. 3.15). An interesting aspect, which is not further discussed here, is that the more than twofold increase of the specific concentration of H-NS during the transient period from the batch phase to the fed-batch phase. This is interesting, as it confirms the observations of Dersch et al. (1993) and Falconi et al. (1996), who have observed that the level of H-NS increases in the early stationary growth phase, whereby the control mechanism seems to be based on a sensitive regulation by the concentrations of Fis and H-NS itself (Falconi et al., 1996).

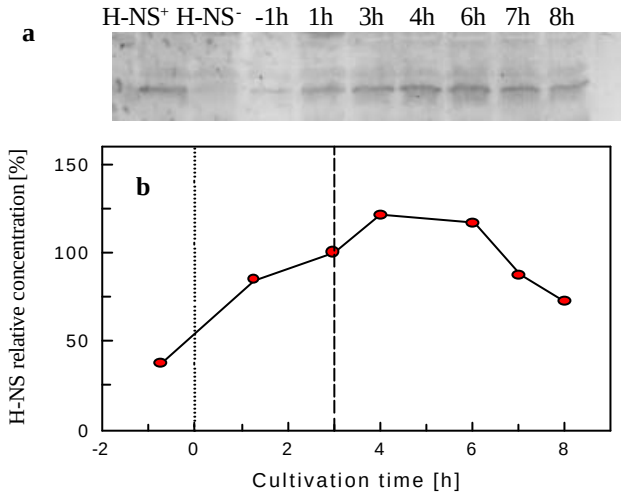


Figure 3.15: Analysis of the H-NS protein during different phases of a glucose limited fed-batch fermentation of *E. coli* RB791 pKK177glucC pUBS520 with induction by 1 mM of IPTG (at three hours, interrupted line). Relative levels of H-NS were determined by immunoblot analysis (**a**). Same cell concentrations according to OD_{500} were applied to each lane. Samples of MC4100 *hns*$^{+}$ and PD32 *hns*$^{-}$ were applied as a reference. Bands representing H-NS were quantified densitometrically (**b**), and the data obtained were normalized for the value determined at the point of induction (sample 3). The feeding was started at zero hours (dotted line).

Furthermore, the loss of DNA condensation could be also caused by DNA strand breaks or DNA damage, which should be connected to the induction of the SOS regulatory network. The expression of the genes which belong to this DNA damage-inducible network are controlled by a complex circuitry involving the RecA and LexA proteins. LexA acts thereby as the repressor of more than 20 genes, including its own *lexA* gene. As the SOS response is connected to a cleavage of the LexA protein by RecA, the typical response after SOS induction is connected to a transient decrease of the LexA level. Indeed, a rapid decrease of the LexA level was found directly after induction of the

α-glucosidase, which indicates DNA damage (see Fig. 3.16). However, LexA is not coming up again, as it does in a non-induced cell, in which DNA damage is induced by DNA damaging agents (see Sassanfar and Roberts, 1990). In contrast to our expectation, the LexA protein was found remaining at the low level and not coming up. The light increase in the last sample (Fig. 3.16a) is related to the beginning up-growth of a plasmid-free cell population, which, of course, had the normal level of LexA and contributes to about 5 % of the total cells in the culture at this time (compare to Fig. 3.3c).

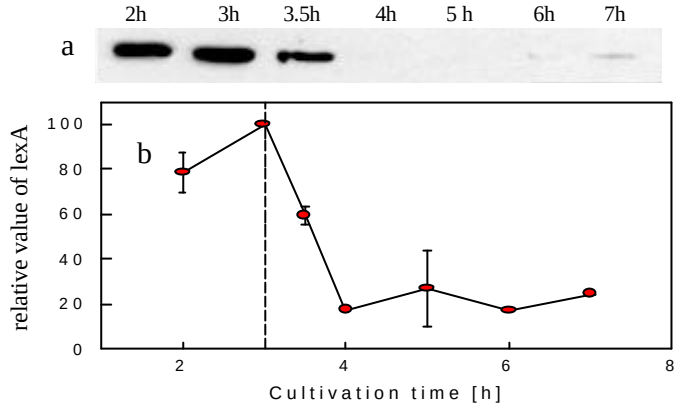


Figure 3.16: Analysis of the LexA protein during a glucose limited fed-batch fermentation of *E coli* RB791 pKK177glucC pUBS520 with induction by 1 mM of IPTG (at three hours, interrupted line). Relative levels of LexA were determined by immunoblot analysis (**a**). Same cell concentrations according to OD_{500} were applied to each lane for demonstration. Bands representing LexA were quantified densitometrically (**b**), and the data obtained were normalized for the value determined at the point of induction. The feeding was started at zero hours.

3.2.5 The energy situation following induction of α-glucosidase

The maintenance of the DNA superhelicity is an energy dependent process and has been shown to be connected to ATP/ADP ratio (van Workum et al., 1996). Therefore, the ATP concentration and the energy charge (EC) in the cell were determined to check the question whether a change in ATP and EC after induction of the α-glucosidase could cause the relaxation of the DNA.

Neither ATP, nor ADP and AMP showed a significant change in their cellular levels in a control fermentation of *E. coli* RB791 pKKglucC pUBS520 without induction. The energy charge (EC) varied in a range between 0.8 and 0.95 and the sum of the adenosine phosphate concentration (AXP) did not change significantly (Fig. 3.17a-f).

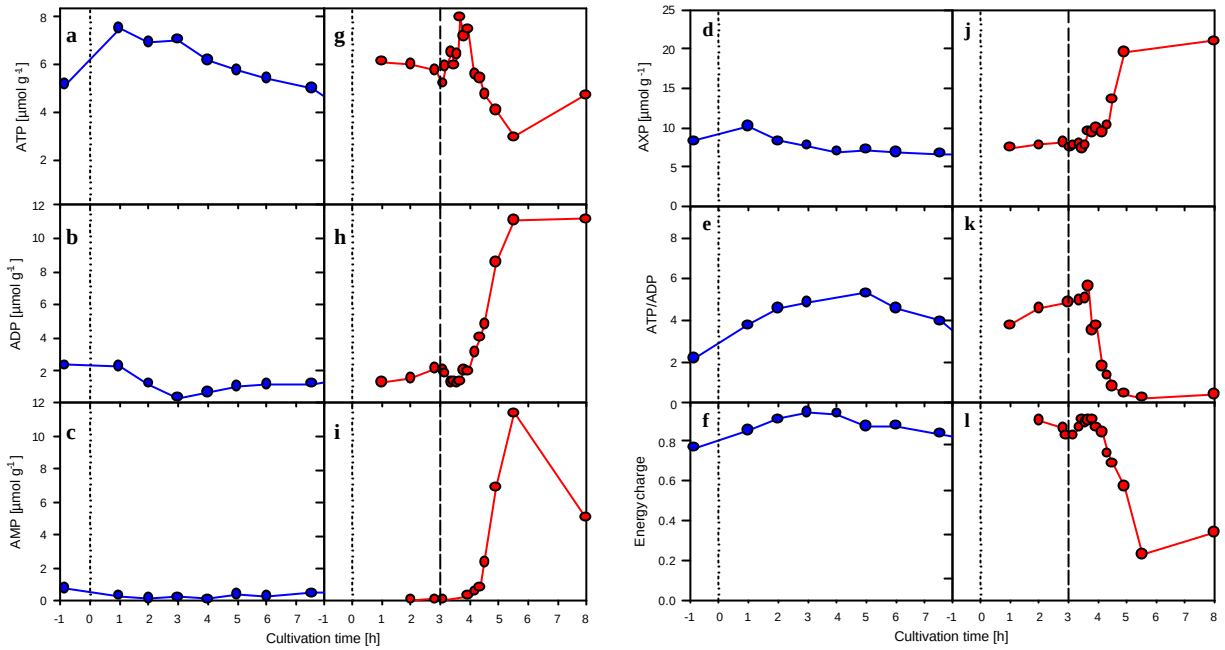


Figure 3.17: The level of adenosine nucleotides and energy charge during fed-batch fermentation of *E. coli* RB791 pUBS520 in dependence on induction of the α-glucosidase (**a-f**: no induction, **g-l**: induction by addition of IPTG). The calculation of ATP/ADP ratio before induction was based on the mean value of the two fermentations (Fig. e, k).

In the contrast to the control fermentation, the adenosine pool showed significant changes following induction of the α-glucosidase (Fig. 3.17g-l). First, the ATP level slightly increased within one hour, which was connected to an increased respiration (qO_2, see Fig. 3.18) and a decreased DOT (Fig. 3.49a). However, at about 1.25 hours after induction respiration began to decrease, and from this point the ATP concentration also decreased and finally reached a very low level, which was only about 30 % of the pre-induction level. This considerable decrease is connected to a rapid increase of the ADP and AMP levels, which in the sum were higher than the sum of adenosine phosphates before induction. The high increase of ADP and AMP cannot be explained only from the decrease of ATP, but might be triggered by RNA degradation, as indicated by S8 immunoblot analysis (Fig. 3.19), which is similar to the system earlier described by Dong et al. (1995). A substantial amount of the ribosomes was also degraded in this α-glucosidase production system after induction. The rise of the lower phosphorylated nucleotides had a strong impact on the energy charge, which decreased below 0.3. Furthermore, the levels of AMP and ADP do not only increase intracellularly, but both nucleotides are also found in significant amounts (about 50 %) in the extracellular medium. Even ATP was detected in minor amounts in the cultivation broth. In conclusion, it could be assumed that the decrease of the ATP level but especially the decrease of the energy charge contribute to the loss of superhelicity.

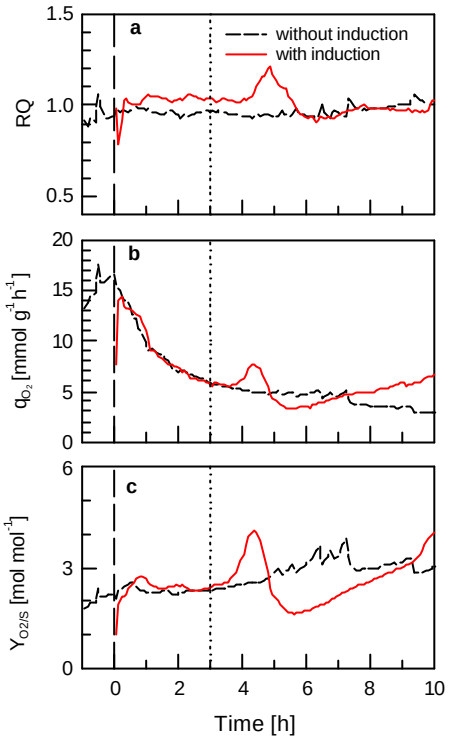


Figure 3.18: Comparison of the respiratory quotient (RQ, **a**), oxygen uptake rate (q_{O2}, **b**), and yield of oxygen per glucose ($Y_{O2/S}$, **c**) between fed-batch cultures of *E. coli* RB791 pKK177glucC pUBS520 without induction (----) and with induction (—) by 1 mM IPTG (dotted line).

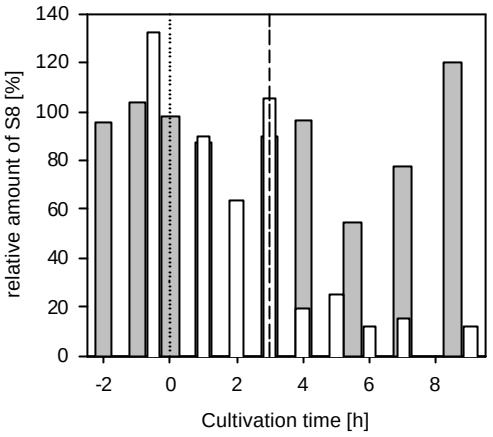


Figure 3.19: Concentration of S8 protein in glucose limited fed-batch fermentations of *E. coli* RB791 pKK177glucC pUBS520 with induction by 1 mM of IPTG at three hours after feeding start and without induction (filled column, as control). The data obtained were normalized for the value determined at the point of induction. The feeding was started at zero hours.

3.2.6 Inhibition of glucose uptake rate after overexpression of recombinant genes

As discussed before, it is only possible that a plasmid-free population grows up from a very low level within a few hours in a glucose limited fed-batch if this population can grow with approximately μ_{max}. Therefore, one should assume that the glucose uptake rate of the induced cells is significantly reduced in comparison to a non induced culture, where never plasmid-free cells were coming up. This hypothesis was checked by measuring the maximum glucose uptake rate during different phases of the fed-batch fermentations.

An experiment was designed to determine whether the maximum glucose uptake rate decrease after overexpression of a recombinant protein (Fig. 3.20). In glucose limited fed-batch fermentations q_{smax} can be easily determined by addition of a glucose pulse and measuring the consumption of glucose by rapid sampling. The glucose uptake was connected to increased respiration, which was obvious by a declining DOT (Fig. 3.21). When the added glucose amount was so high to saturate the cellular uptake system(s), the DOT approached a level that corresponded to the q_{O2} at the maximum glucose

uptake rate or to the respiratory capacity if this was lower than the maximum glucose inflow. Then the DOT kept at this value as long as the glucose uptake is constant. Only if the glucose approaches a level which ia not saturating the uptake system(s) (tR), the DOT is rising. For *E. coli* this DOT rise is very rapid at cell concentrations in the g cell dry weight per liter range due to the very low Ks value for glucose that is in the order of a few mg L^{-1} (Neubauer et al., 1995).

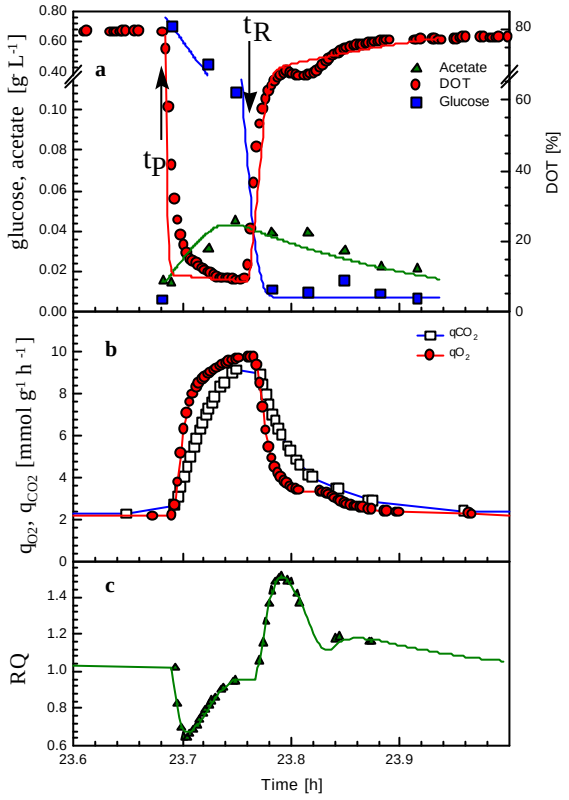


Figure 3.20: Responses following a glucose pulse during a glucose limited fed-batch. (**a**): Glucose (—□—), acetate (—▲—) and DOT(—●—). The symbols indicate measured values, the lines correspond to simulation results. (**b**): q_{O2} (—●—) and q_{CO2} (—□—), (**c**): Respiratory quotient (—▲—). The glucose pulse is indicated by an arrow.

From the DOT curve q_{smax} can be easily calculated by the time interval between the response of the DOT signal after the glucose pulse and tR, as expressed by the formula:

$$qsmax = \frac{[Glc + \int_{tP}^{tR} (F*Si)]}{V*X*(tR-tP)} \qquad (24)$$

The application of this calculation of the q_{smax} only by the DOT signal was restricted to the following conditions: (1) It can be only applied if the growth is limited by glucose, and (2) if there is an respiratory capacity which allows the decrease of DOT.

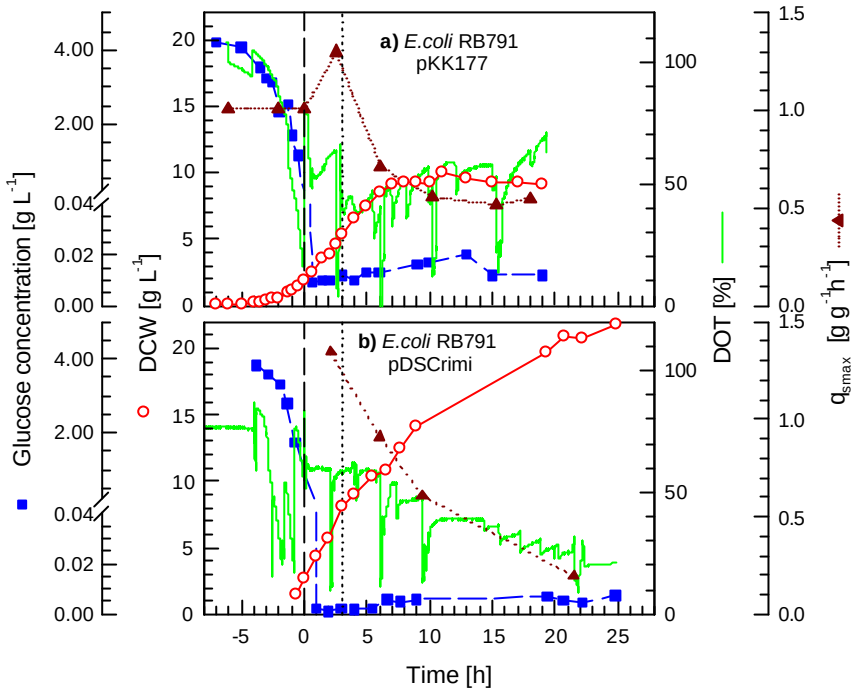


Figure 3.21: Cell growth, glucose consumption, DOT and q_{smax} during different phases of fed-batch cultivations of *E. coli* RB791 with pKK177glucC (**a**) or pDSCrimi (**b**) with induction of 1mM IPTG (dotted line). Feeding start at zero hour (interrupted line).

The calculated q_{smax} was about 1.1 to 1.3 g g^{-1} h^{-1} during the batch phase. This capacity for the uptake of glucose did not decrease but even slightly increased during the shift from glucose unlimited to limited growth. However, after induction of the α-glucosidase, the q_{smax} decreased to about 50 % of the q_{smax} of the batch phase in the cultivation of *E. coli* RB791 pKK177glucC (Fig. 3.21a). q_{Smax} was even more reduced in the strain *E. coli* RB791 pKK177glucC pUBS520 (Fig. 3.22). This capacity for uptake of glucose decreased to about 50 % of the maximum glucose uptake rate of the batch phase during control fermentation without induction independent on the growth rate. After induction of the α-glucosidase a rapid reduction in the maximum glucose uptake rate to about 24 % of the q_{smax} of the batch phase was determined. The analysis of the residual glucose in the cultivation medium resulted in a q_{smax} of 0.37 g g^{-1} h^{-1}. Interestingly, q_{Smax} was also inhibited in *E. coli* RB791 pDSCrimi fed-batch cultivations after induction of CRIMI , but a lower rate than in the α-glucosidase system (Fig. 3.21b).

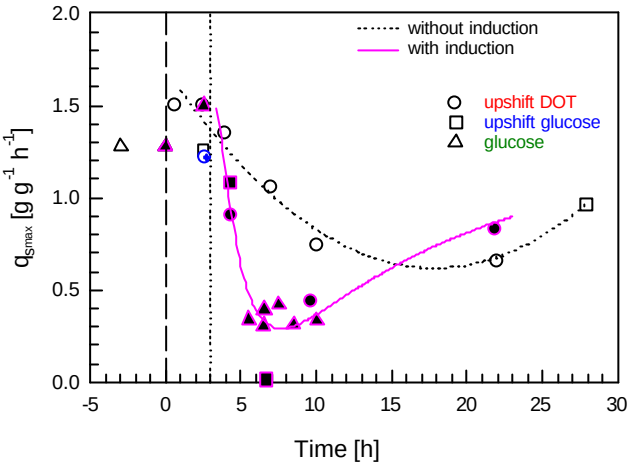


Figure 3.22: Comparison of q_{smax} during different phases of fed-batch cultivations of *E. coli* RB791 pKK177glucC pUBS520 with induction (—) and without induction (----). Feeding start (vertical interrupted line) and induction with 1mM IPTG (vertical dotted line) are indicated.

These results indicated that the specific capacities for glucose uptake (q_{scap}) and oxygen consumption (q_{ocap}) were not constant during the course of a fed-batch fermentation. Both could be significantly reduced by the production of recombinant proteins, which were important in consideration to the up-growth of plasmid-free cells. The residual glucose in the medium caused an unlimited growth of the plasmid-free population that can reach a considerable level of the total population within a few hours. The knowledge about the kinetics of the decrease of q_{smax} and q_{O2} was used in a model to simulate the up-growth of the plasmid-free population on the basis of monod kinetics. The measured growth parameters can be correctly fitted by this model (also see Fig. 4.3 and Table 4.3).

3.3 Stress responses after induction of recombinant gene expression

A decrease of the specific glucose uptake capacity after induction of α-glucosidase would result in severe carbon and energy starvation if glucose is the only carbon source. Furthermore, the inhibition of glucose uptake could be suggested to induce glucose starvation specific cellular responses, such as the cAMP/CRP mediated response, the general stress response and the stringent response. In this section, the effect of the overexpression of heterologous genes (α-glucosidase and CRIMI) in *E. coli* on the level of different stress response regulators was investigated.

3.3.1 Level of the stringent response regulator ppGpp

It is well established that the general stress response and the stringent response are important adaptation strategies of *E. coli* in order to survive adverse environmental conditions, such as prolonged starvation (McCann et al., 1991). Confirming the results of Andersson et al. (1996), Fig. 3.23a shows that the level of the alarmone ppGpp was highly elevated in the transient phase from unlimited growth to glucose limitation in fed-batch fermentations, but decreased also fast and remained at a higher level during the glucose limited growth phase than in the unlimited growth phase.

When samples were collected during the fermentation and exposed to glucose starvation (see Fig. 3.24), ppGpp concentration raised within 60 sec to about 0.3 µmol g^{-1} DCW confirming the results of Neubauer and coworkers (1995a) that changes in the glucose availability by changes in the feed rate are immediately responded by an appropriate change of the ppGpp level (also see Andersson et al., 1996b).

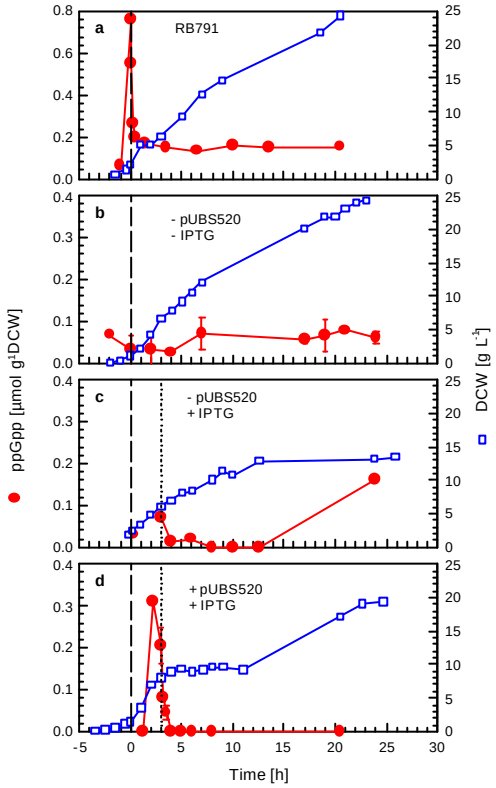


Figure 3.23: Cellular concentration of ppGpp (●, µmol g^{-1} DCW) and the dry cell weight (□, g L^{-1}) in fed-batch cultivations of *E. coli* RB791 pKK177glucC with and without pUBS520. **(a)** *E. coli* RB791; **(b)** *E. coli* RB791 pKK177glucC without induction; **(c)** *E. coli* RB791 pKK177glucC with induction; **(d)** *E. coli* RB791 pKK177glucC pUBS520 with induction. The time of induction is indicated by dotted line at 3 h after feed start. ppGpp is suggested to increase also in **(b)** and **(c)** within three hours after feeding start, however, no data were analyzed in this time frame for these two fermentations.

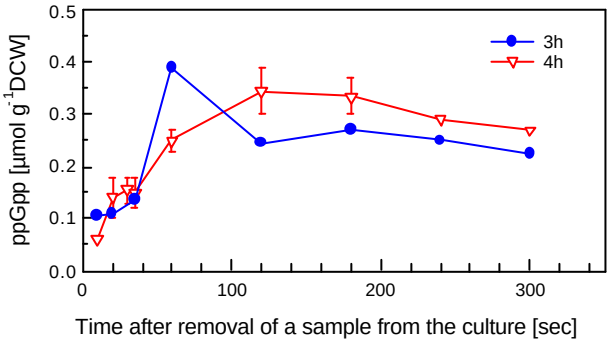


Figure 3.24: ppGpp concentration after removal of a sample from the culture (at time zero). The two different samples were taken at the time 3 h and time 4 h from the glucose limited fed-batch fermentation of *E. coli* RB791 without plasmid.

As described in section 3.2.6, we found that the cellular glucose uptake capacity was strongly inhibited after induction of the α-glucosidase, and concluded that the cells would run by this into glucose starvation. Probably, this would also result in a ppGpp response, which has been shown for human SOD by the group of K. Bayer (Cserjan-Puschmann et al., 2000). Thus, similar to the

induction of a heat shock like response following overexpression of a recombinant gene which has been described for many proteins by many authors (Goff & Goldberg, 1985, 1987; Kosinski & Bailey, 1991; Kosinski et al., 1992), the stringent response and following, by the positive impact of ppGpp on rpoS expression, also the general stress response might be induced after strong induction of a recombinant gene.

In order to answer the question whether the stringent response alarmone ppGpp plays any role in the growth inhibition and decrease of colony forming units after induction of the *tac*-promoter directed overexpression of the α-glucosidase, the level of ppGpp after induction was analyzed by HPLC and is shown in Fig. 3.23c,d. Similar to the control cultivation with the plasmid-less strain, there was an increase of ppGpp in all cultures, when they run into glucose limitation (shown only for RB791 pKK177glucC pUBS520 in Fig. 3.23d). After the peak, the level of ppGpp increased to about 200 % of the value of the batch phase in the glucose limited growth phase. However, after induction of the α-glucosidase expression, ppGpp was found to drop below the detection limit within two hours (Fig. 3.23c,d), independently, whether the strain contained the pUBS520 plasmid or not. The results suggest that no stringent response is induced after overproduction of α-glucosidase in both systems with and without plasmid pUBS520.

3.3.2 The σ^S-related general stress response

Level of σ^S after induction of recombinant α-glucosidase. The *rpoS* encoded sigma factor σ^S (σ^{38}, RpoS, KafF) is a master regulator which is induced in response to a variety of environmental stress conditions that include starvation for various nutrients, entry into stationary phase, high osmolarity, acid shock, and high or low temperature (Lange & Hengge-Aronis, 1991; Hengge-Aronis, 1996a,b, 1999). Furthermore, it has been proved that rpoS gene expression is positively regulated by ppGpp (Gentry et al., 1993; Lange et al., 1995). Therefore, it is worth to see on the level of σ^S in connection to the surprising result of the ppGpp decrease after induction of α-glucosidase.

As shown in Figure 3.25a and c (see also Fig. 3.26a,c), the concentration of σ^S increased, accordingly to the ppGpp level, in the control fed-batch cultures at the point when the culture run into glucose limitation independent, whether the strain contains a plasmid or not. A 1.5 to two-fold higher level of σ^S than in the batch phase was maintained up to the end of the cultivation if no induction was performed, although σ^S slightly decreased to about 50 % of the maximum value in some cases.

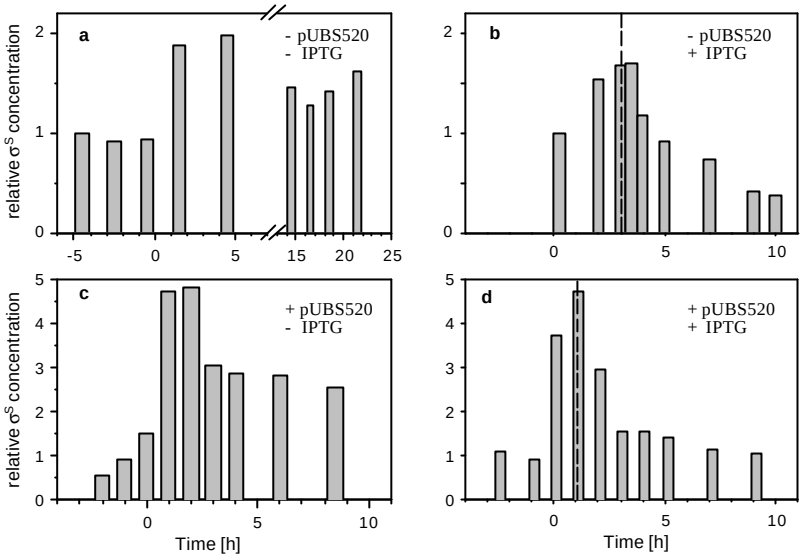


Figure 3.25: σ^S in fed-batch cultivations of *E. coli* RB791 pKK177glucC with and without pUBS520. **(a)** *E. coli* RB791 pKK177glucC without induction; **(b)** *E. coli* RB791 pKK177glucC with induction; **(c)** *E. coli* RB791 pKK177glucC pUBS520 without induction; **(d)** *E. coli* RB791 pKK177glucC pUBS520 with induction. Relative σ^S levels (grey bars) were normalized for the values determined during exponential growth after immunoblot analysis and densitometrical quantification. The time of induction is indicated by an interrupted line. Time zero corresponds to the start of glucose limitation according to the DOT signal (not shown).

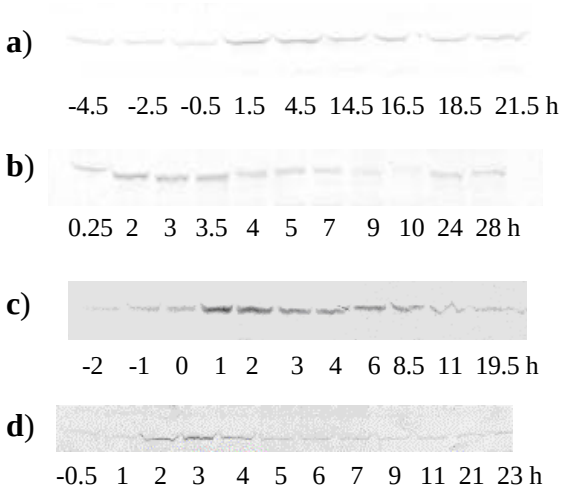


Figure 3.26: Immunoblots of the cellular levels of σ^S in glucose limited fed-batch fermentations of *E. coli* RB791 pKK177glucC with and without pUBS520. **(a)** *E. coli* RB791 pKK177glucC without induction; **(b)** *E. coli* RB791 pKK177glucC with induction; **(c)** *E. coli* RB791 pKK177glucC pUBS520 without induction; **(d)** *E. coli* RB791 pKK177glucC pUBS520 with induction. A time of zero hours is related to the feed start. IPTG was added at 3 h to the cultivations of panels **(b)** and **(d)**.

After IPTG was added to cultures of *E. coli* RB791 pKK177glucC pUBS520 the concentration of σ^S significantly decreased to only 20 % of the value at the induction point within one hour (Fig. 3.25d). The reduction of σ^S was also measured in cultures with *E. coli* RB791 pKK177glucC without pUBS520 (Fig. 3.25b). In accordance to these results, no increase of σ^S level was measured

after addition of IPTG in shake flask cultures of both strains where the level of σ^S was closely to the detection limit at the point of induction (data not shown).

Effect of a *clpP* mutation on the overexpression of the α-glucosidase. Opposite to our expectations, the analysis of stress response regulators showed that neither ppGpp nor σ^S is induced in response to the growth inhibition caused by overproduction of the α-glucosidase. Rather, the concentrations of σ^S and ppGpp decreased to the detection limit. According to the results of Schweder and coworkers (1996) that σ^S is an unstable protein and is degraded by the ClpXP protease, the effect of a *clpP*-deficient mutation on the overproduction of recombinant α-glucosidase in *Escherichia coli* was investigated, as one could suggest that the decreased level of σ^S could be responsible for the loss of cell viability after induction of the α-glucosidase.

In accordance to the results of Schweder and coworkers (1996) a high level of σ^S was found by immunoblot analysis in the *clpP* mutant strain before, and also after induction with IPTG (Fig. 3.27c). The analysis of the colony forming ability showed a significant higher viability of the *clpP*-deficient strain in comparison to the wildtype (Fig. 3.27a). By assumption that the colony forming ability is decreased by a first order kinetics, this differences are related to a specific death rate k_D of 0.25 ± 0.08 h^{-1} for the *clpP*$^+$ wildtype strain and 0.06 h^{-1} for the *clpP* mutant. Generally the *clpP* mutant grew significantly more slowly (μ_{max}=0.51 h^{-1}) than the wildtype (μ_{max}=0.79±0.05 h^{-1}), possibly due to higher level of σ^S. This higher level of σ^S in the *clpP* mutant further increased after glucose limitation during the transient to glucose limited growth and remained at a high level during the fed-batch phase of growth. This high level was not influenced by induction of the α-glucosidase in the *clpP* mutant (Fig. 3.27c). From this result one could suggest that the higher σ^S level is responsible for the better survival. However, the high level of σ^S and the better survival in the *clpP* mutant did not improve the yield of the α-glucosidase (Fig. 3.27b). On the contrary, the α-glucosidase level in the *clpP* mutant was clearly lower than in the wild type. The corresponding specific production rates (q_p in g per g cell dry weight and hour) were 15 mg g^{-1} h^{-1} for the *clpP*$^+$ wildtype strain and 4 mg g^{-1} h^{-1} for the *clpP* mutant. Interestingly, the growth of the culture was not inhibited after induction in this mutant, but was approximately the same as in the culture without induction.

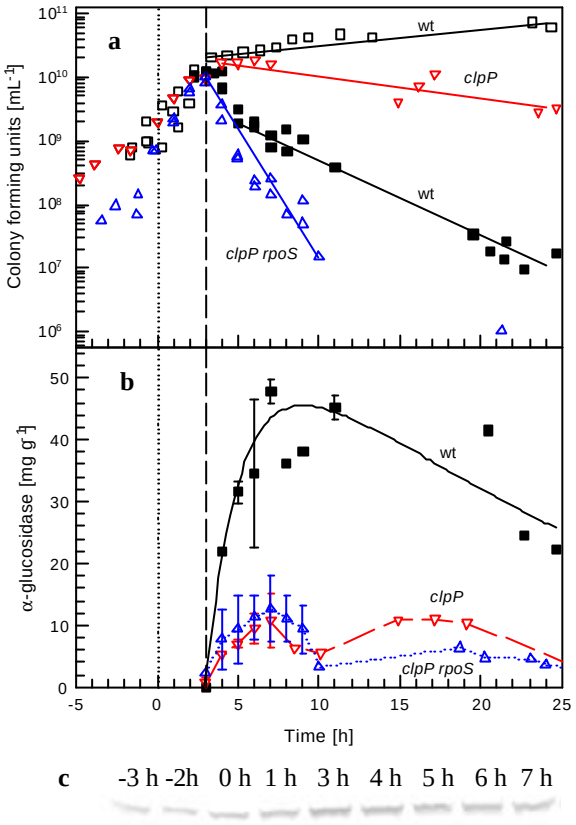


Figure 3.27: Colony forming ability **(a)** and α-glucosidase production **(b)** of *E. coli* RB791 pKK177glucC pUBS520 (□ without induction; ■ with induction) and of the corresponding *clpP* (▽) and *clpP rpoS* (△) mutants in glucose limited fed-batch cultivations. The feeding start is indicated by a dotted line and the point of IPTG addition is shown by the interrupted line. The data origin from two independent fermentations of each strain. **(c)** Immunoblot of the cellular levels of σ^S in the *clpP* mutant *E. coli* RB791P pKK177glucC pUBS520 with induction.

Furthermore, experiments with a *clpP rpoS* double mutant (RB791PS) were performed in order to answer the question whether the high level of σ^S competes with σ^{70} for the available RNA polymerase and thus is responsible for the lower production of α-glucosidase in the *clpP* mutant. In the *clpP rpoS* double mutant μ_{max} was very similar to the growth rate of the wild type. But in contrast to the wild type, the double mutant, similar to the *clpP* mutant, showed a clearly longer lag phase of growth (also see Damerau and John, 1993). Following induction, the *clpP rpoS* double mutant showed a strong loss of viability (k_d=0.79 h^{-1}), although surprisingly the yield of α-glucosidase was not as high as in the cultivation of the wildtype, and only slightly higher than in the *clpP* mutant (Fig. 3.27b). However, the analysis of α-glucosidase mRNA showed that the induction of the recombinant gene was the same in the double mutant as in the wild type, but lower in the clpP mutant (see Fig. 3.29f). These results indicate that σ^S competes with σ^{70} for the available RNA polymerase in accordance to our suggestions, but on the other hand, that σ^S is also important for the survival of the cells after stress and recombinant gene induction.

Effect of CRIMI overproduction on the general stress response. In contrast to the case of α-glucosidase overproduction where no increase of σ^S was determined after addition of IPTG, the induction of recombinant CRIMI caused a strong increase of σ^S (Fig. 3.28).

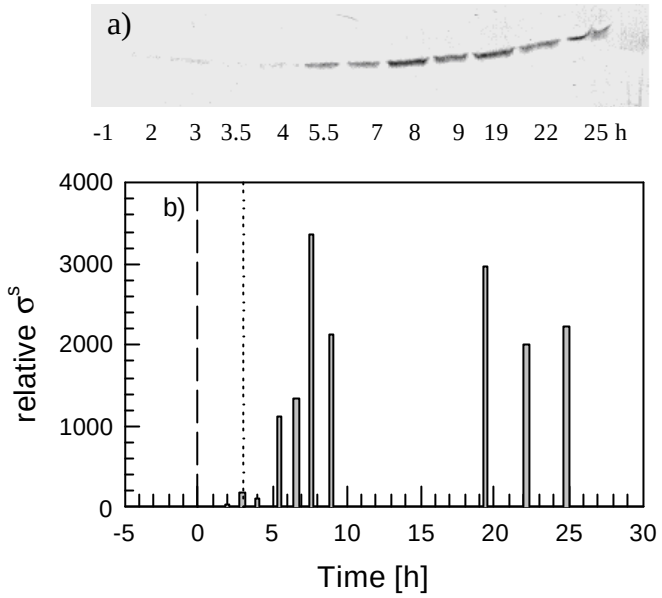


Figure 3.28: Cellular σ^S response (**a**: immunoblot, **b**: relative σ^S value) during glucose limited fed-batch culture of *E. coli* RB791pDSCrimi. A time of zero hours is related to the feed start (interrupted line). IPTG was added at 3 h to the cultivation (dotted line).

After the addition of IPTG to the culture, the concentration of σ^S increased markedly within four hours, clearly demonstrating a rapid general stress response to the overproduction of the heterologous protein CRIMI. Furthermore, the level of σ^S remained at a higher level up to the end of the cultivation. Although both products, α-glucosidase and CRIMI, were accumulated to more than 10 % of total cell proteins and to a major part as inclusion bodies, significant differences were detected in connection to the influence of the recombinant production on the cellular growth and cell survival. The results suggest that induction strength on the transcriptional level and the strength of the ribosome binding site, even the gene codon usage of a recombinant gene influence the behavior of stress signals. Moreover, an appropriate general stress response may be important for maintenance of protein synthesis over a longer time interval in fed-batch fermentations

3.3.3 Comparison of mRNA levels of genes controlled by different $_S$ factors

As described above, the observed higher σ^S level in the *clpP* mutant was suggested to compete with σ^{70} for the available RNA polymerase molecules, which consequently would lower the σ^{70}-promoter directed expression of the α-glucosidase in the *clpP* mutant. Therefore, the mRNA levels of different σ^S and σ^{70} dependent genes were analyzed by investigating the mRNA level of the σ^{70}-dependent α-glucosidase gene, the *rpoS* gene and the σ^S-dependent gene *osmY*. The σ^{70}-dependent *rpoA* mRNA coding for the α-subunit of the RNA polymerase was used as control.

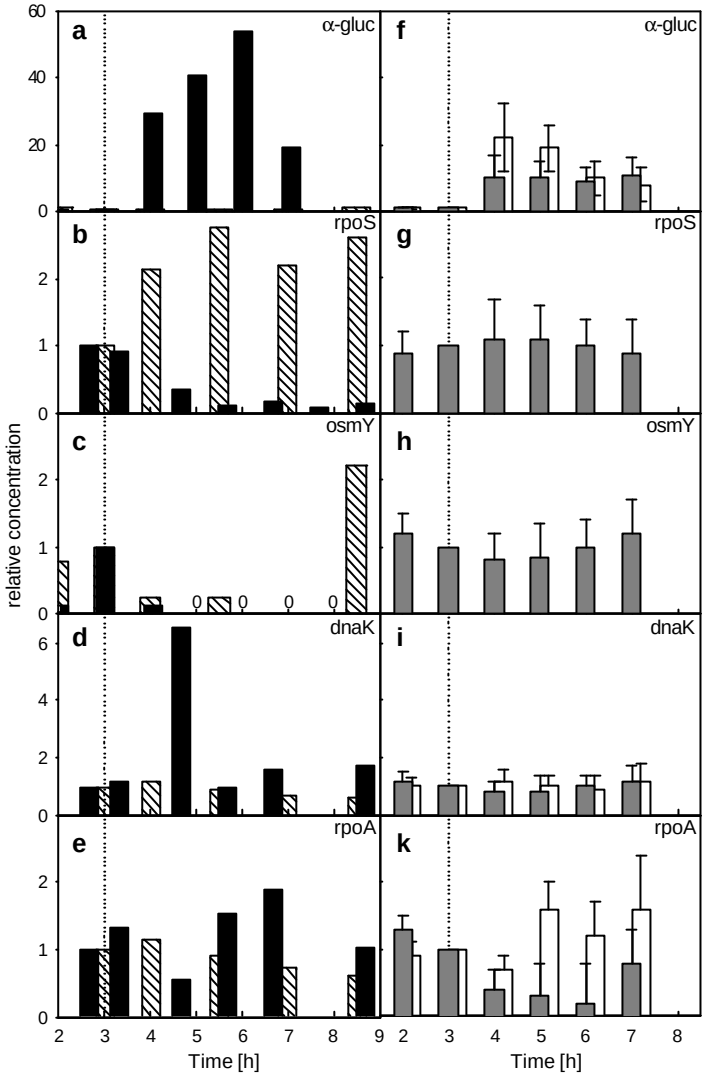


Figure 3.29: Concentration of different mRNA's in glucose limited fed-batch cultures of *E. coli* RB791 pKK177glucC pUBS520 **(a-e)** with induction (black bars) and without induction (reference, striped bars) in comparison to corresponding cultivations with the RB791P (gray bars) and the RB791PS (white bars) strains **(f-k)**. The concentrations were calculated on the basis of the total RNA and normalized to the values determined short before induction. The zero values in graph **(c)** of *osmY* mRNA belong to the fermentation with induction of α-glucosidase. No *osmY* mRNA could be detected in the *clpP rpoS* mutant. The time of IPTG addition is indicated by a dotted line.

Fig. 3.29b shows that the level of the *rpoS* mRNA is drastically reduced following induction of the α-glucosidase production in the wild type. This differs from the control cultivation without induction where the level was higher during the fed-batch phase than the exponential growth phase. The decrease in the *rpoS* mRNA level after induction of the *tac*-promoter is consistent with the decrease in the σ^S level, and the low ppGpp level. Furthermore, the low level of σ^S, consequently negatively influences the expression of genes, which are positively controlled by σ^S, such as *osmY*. After induction of the α-glucosidase, the amount of *osmY* mRNA decreased below the detection limit and

did not rise to the end of the cultivations (19 hours after induction, data not shown). In contrast, the *osmY* mRNA level in the control cultivation without induction increased with a delay of some hours after σ^S (Fig. 3.29c), possibly because it is negatively controlled by further factors, such as cAMP and Lrp (Lange et al., 1993). The level of the *osmY* mRNA was very high in the *clpP* mutant in agreement with the high σ^S level in this strain. It appears that *osmY* is exclusively transcribed by the σ^S RNA polymerase holoenzyme. No *osmY* mRNA could be detected in a *rpoS* mutant (data not shown).

In order to prove whether the competition of σ^S and σ^{70} for RNA polymerase lowers the induction of α-glucosidase at the mRNA level in the *clpP* mutant, the α-glucosidase mRNA level in the *clpP rpoS* double mutant was investigated. In this double mutant, α-glucosidase mRNA increased within one hour after induction to approximately the same level as in the wild type, which would be consistent with the hypothesis. However, in contrast to the wild type, the amount of the α-glucosidase mRNA decreased more quickly. Whereas the highest level of α-glucosidase mRNA was detected in the wildtype three hours after induction, the value in the *clpP rpoS* double mutant was highest one hour after induction.

rpoA mRNA was measured as a control for a host vegetative gene which is transcribed by σ^{70} RNA polymerase holoenzyme. The amount of this mRNA was influenced only to a minor extent by the induction of the α-glucosidase. However, within approximately two hour after induction the concentration of *rpoA* mRNA drops to 50 %, corresponding to a high synthesis of *dnaK* mRNA (Fig. 3.29d) and *groEL* mRNA (not shown here, see Jürgen et al., submitted manuscript) at this time. Thus, one could suggest a competitive effect of σ^{32} shortly after induction, which was also supported by the 2D PAGE analysis of the samples from the same fermentations where a higher level of DnaK and GroEL was detected after induction of the *tac*-promoter directed overexpression of the α-glucosidase (Jürgen et al., submitted manuscript). This is in accordance with a number of earlier studies from other groups (Goff & Goldberg, 1985, 1987; Kosinski & Bailey, 1991; Kosinski et al., 1992), which showed that the heat shock response is induced by recombinant protein production. Interestingly, an increase of the *dnaK* mRNA level was only found in the wild type, but not in the *clpP* and *clpP rpoS* mutants, possibly due to the low synthesis of α-glucosidase in both mutants, because it is known that the incorrectly folded product induces the heat shock like response in recombinant production systems.

3.3.4 Level of cAMP in fed-batch fermentations of *E. coli*

cAMP is a further nucleotide with a regulatory role in the cellular response to glucose limitation and starvation. Although the phenomenon of cAMP formation by the adenylate cyclase in connection to glucose availability and its influence in the transcriptional gene regulation in connection to CRP are intensively studied, the knowledge is low concerning the mechanisms of cAMP efflux and reconsumption and possible consequences for the cell physiology. However, it is a well-known phenomenon that the cellular level of cAMP dramatically increases after glucose exhaustion and is kept at intermediate levels if glucose concentrations are low (Saier, 1996). cAMP enables the cAMP receptor protein (CRP, *CAP*) to binding at *crp* regions triggering the stimulation of several genes (Ebright et al. 1989). Although cAMP is a very fast reacting molecule in the cell, it is chemically stable and can be easily determined in medium samples. According to Matin (1982), almost all (over 99.9%) of the cAMP made by *E. coli* grown at several dilution rates in a chemostat was excreted into the medium. They suggested that extracellular cAMP possibly also has a role in sensing cell density or other intercellular communication. Therefore, cAMP was analyzed in the fermentation medium, to investigate whether differences can be determined from various fermentations and to which extent the cAMP data correlate to the other stress response regulators.

Extracellular cAMP analysis after overproduction of $_{a}$-glucosidase. Figure 3.30 illustrated the kinetics of the medium cAMP concentration during fed-batch cultivations of *E. coli* RB791 pKK177glucC with and without pUBS520. After total consumption of initial glucose the level of cAMP in medium began to increase in both strains, although the accumulation rate was different and ranged from 0.46 µmol L^{-1} h^{-1} to 2.32 µmol L^{-1} h^{-1} within the first 4 hours of the glucose limited phase. In the non-induced cultivation of *E. coli* RB791 pKK177glucC pUBS520 cAMP was accumulated in the cultivation medium to a concentration of 9 µM (Fig. 3.30a). Similarly, no significant difference was observed in the cultivation of *E. coli* RB791 pKK177glucC after induction (Fig. 3.30b). The efflux of cAMP maintained a constant level and the specific cAMP concentration in medium was at an intermediate level of 0.8 ± 0.1 µmol g^{-1} DCW.

Surprisingly, the medium cAMP concentration started to decrease approximately one hour after induction of the α-glucosidase in cultures with *E. coli* RB791 pKK177glucC pUBS520 (Fig. 3.30c). The cAMP was reconsumed with a rate of 0.97 µmol L^{-1} h^{-1}. At the same time the increase of the glucose concentration in the medium (Fig. 3.1h) can be detected. Furthermore, the volumetric concentration of cAMP increased again from 11 hours after feed start, where glucose became

limiting again due to up-groeth of plasmid-free cells. However, from this time period only the up-coming plasmid-free cells were supposed to produce cAMP, as the concentration was only increasing very slowly. This suggestion is based on the calculation of the cAMP accumulation by assuming the same rate of cAMP efflux as after the batch phase, when the culture became glucose limited for the first time.

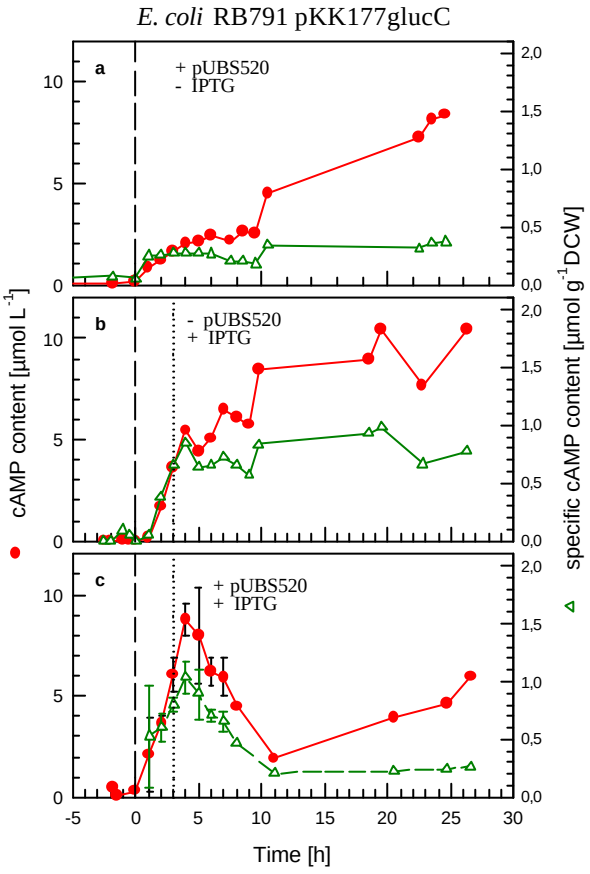


Figure 3.30: Concentration of extracellular cAMP (● µmol L^{-1}; Δ µmol g^{-1} DCW) in glucose limited fed-batch fermentations of *E. coli* RB791 pKK177glucC with and without pUBS520. **(a)** *E. coli* RB791 pKK177glucC pUBS520 without induction; **(b)** *E. coli* RB791 pKK177glucC with induction; **(c)** *E. coli* RB791 pKK177glucC pUBS520 with induction. The interrupted line represents the time of feeding start and the dotted line indicates addition of IPTG.

Influence of recombinant CRIMI product on the concentration of cAMP. Besides a strong increase of σ^S as described in section 3.3.2, overproduction of CRIMI was also connected to a continuous accumulation of cAMP in the culture with a rate of 1.77 µmol L^{-1} h^{-1} that was not changed by the addition of IPTG (Fig. 3.31). The specific concentration of cAMP reached a level of 1.7 µmol g^{-1} DCW. In contrast to the α-glucosidase, the efflux of cAMP was not affected by overproduction of CRIMI.

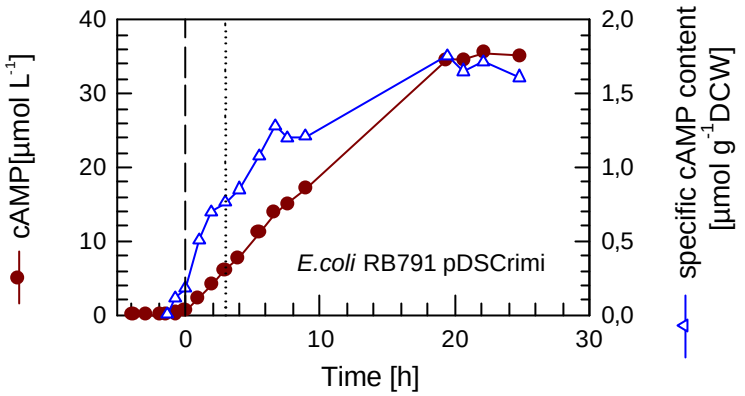


Figure 3.31: Concentration of extracellular cAMP (● µmol L^{-1}; Δ µmol g^{-1} DCW) during glucose limited fed-batch fermentation of *E. coli* RB791 pDSCrimi. The interrupted line represents the time of feeding start and the dotted line indicates addition of IPTG.

Comparison of ppGpp, σ^S and cAMP level in glucose limited fed-batch cultivation of *E. coli* MC4100 relA$^+$. Furthermore, the level of ppGpp, σ^S and cAMP was measured in glucose limited fed-batch cultivation of *E. coli* MC4100 relA$^+$ (Fig. 3.32). The samples were obtained from the cultivations performed by the group of G. Larsson (KTH, Stockholm) within a EU-project (BIO4-CT98-0167) to study cell physiology under fed-batch cultivation conditions. The cultivation was performed to reach a cellular density of 7.5 g dry cell weight per liter in a 10 L fermenter with such a feed protocol that an initial exponential feed was followed by a constant feed. In this non-recombinant fed-batch cultivations, the level of ppGpp reached an approximately constant level during the exponential feed phase and increased slightly after the change to the constant feed (Fig. 3.32a). Fig. 3.32b illustrates the levels of two different cellular sigma factors. The house keeping sigma factor σ^{70} showed no significant changes during the cultivation. In contrast, the level of σ^S increased about twofold from the basal level of cells growing with the maximum growth rate (first sample in Fig. 3.32c). Interestingly, σ^S also tendentiously decreased during the last hours of the fermentation.

The level of intracellular cAMP was determined to be about 0.5 µmol g^{-1} DCW during the exponential feed phase and accumulated to a maximum of 1 µmol g^{-1} DCW during the constant feed phase. Meanwhile, the extracellular cAMP accumulated exponentially to 15 µM in the cultivation medium (Fig. 3.32c). However, if the specific growth rate decreased below 0.05 h^{-1} shortly after the shift from the exponential feeding to the constant feeding, the efflux of cAMP became lower and finally the extracellular cAMP concentration declined. This change in the cAMP accumulation behavior seems fairly well to correlate with the switch from phase two of growth (limitation) to phase three (severe starvation) in the model of different growth phases presented by Chesbro (1990).

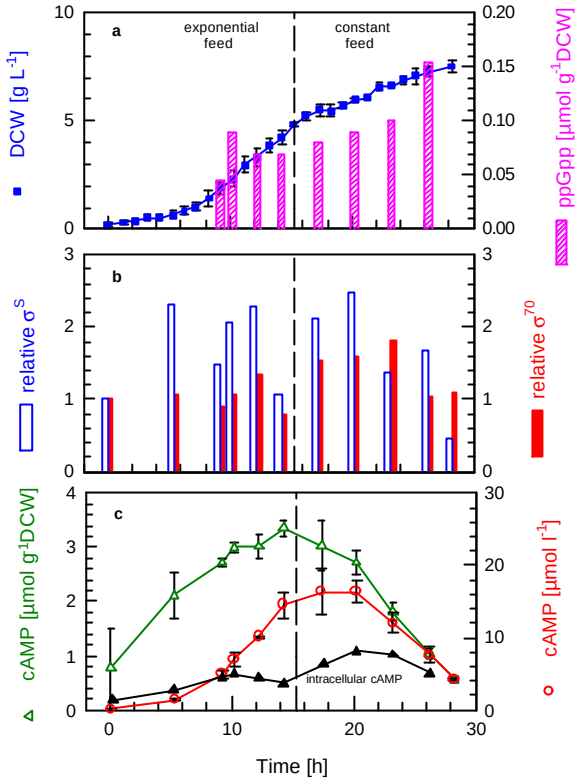


Figure 3.32: Cell growth and the level of stress regulators during glucose limited fed-batch cultivations of *E. coli* MC4100 relA$^+$ in a 10 L fermenter. **a**) Cell growth (■) and ppGpp concentration (bar), **b**) relative σ^S (open bar) and relative σ^{70} (filled bar), **c**) concentration of extracellular cAMP (○ µmol L^{-1}; Δ µmol g^{-1} DCW) and intracellular cAMP (▲ µmol g^{-1} DCW). The interrupted line marks the time point of change from exponential to constant feed. Error bars indicate variations of samples taken from two independent fed-batch cultivations.

3.4 Cell segregation and stress responses in large-scale cultivations

One task of this thesis was to study the cell growth kinetics and physiology during large-scale fermentations which were performed within a EU network project based on integration of microbial physiology and fluid dynamics, in order to develop generalized methodologies and tools to identify critical parameters to be included in new scale-up methodologies for microbial bioprocesses. Here I present the data of large-scale fed-batch fermentations of *E. coli* with and without overproduction of a recombinant protein ZZ (17.7 kDa), a modified B domain of staphylococcal protein A, in industrial 12 m^3 and 30 m^3 scale bioreactors respectively. The major objective of our group was the analysis of cell viability, plasmid stability, energy charge and stress response in large-scale processes.

3.4.1 Large-scale cultivations of *E. coli* W3110

In order to get a comprehensive information about the cell growth and substrate consumption in industrial scale bioprocesses, three fed-batch fermentations of *E. coli* W3110 with different stirrer configurations and feed strategies were performed in the 30 m^3 stirred tank reactor with an initial

working volume of 20 m^3 (see Table 3.2). The growth profiles and on-line data from one of the three similar cultivations are shown in Fig. 3.33 and Fig. 3.34. This fermentation (ET01) was performed with a standard Rushton configuration (see Fig. 3.37) and was fed with glucose to the top of the fermenter, just below the liquid surface. Ammonia for pH-control was added above the liquid surface.

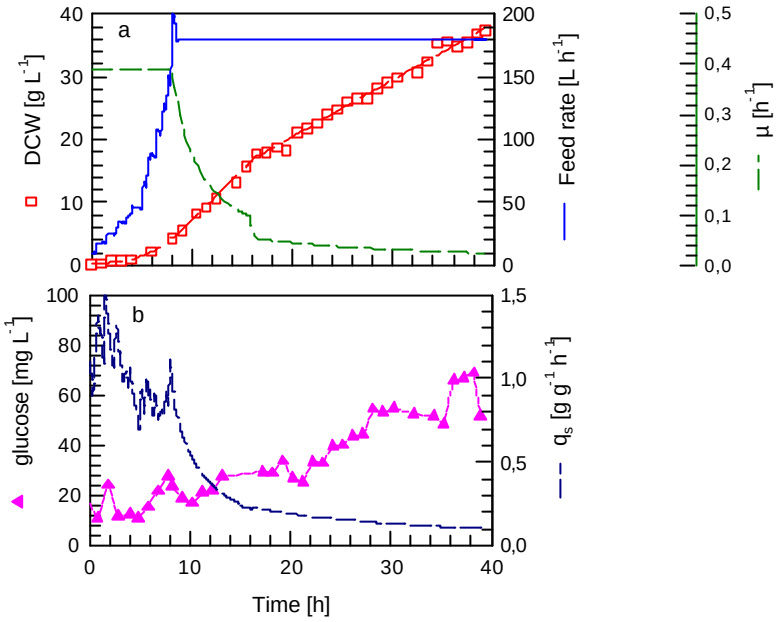


Figure 3.33: Fed-batch cultivation of *E. coli* W3110 in a 30 m^3 bioreactor. **a**) cell dry weight (□), specific cell growth rate (μ, ---) and feed profiles (—); **b**) measured glucose concentration (▲) and specific glucose uptake rate (---). (The glucose data were provided by B. Xu).

During the first phase with exponential feeding the specific growth rate stayed approximately constant at 0.39 h^{-1}. The second growth phase, after switch from quasi exponential feeding to constant feeding at 8 hours, was characterized by a gradually declining specific growth rate (Fig. 3.33a). Within the constant feed phase, the overall biomass yield per glucose was calculated to be 0.28 gg^{-1}. The glucose concentration was low but showed a slightly increasing tendency from 25 mg L^{-1} to 65 mg L^{-1} (Fig. 3.33b). The maximal specific glucose uptake rate was 1.3 g g^{-1} h^{-1}during the first phase of the cultivation.

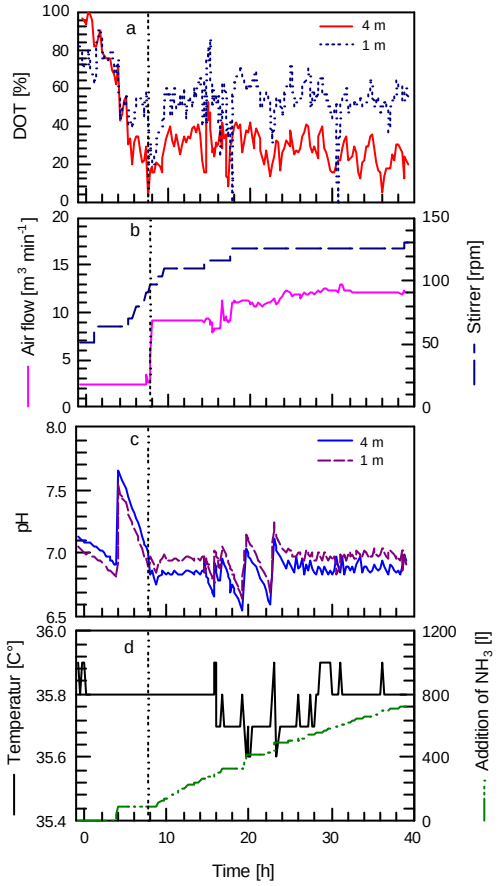


Figure 3.34: On-line measurement during fed-batch cultivation of *E. coli* W3110 in a 30 m^3 bioreactor [ET01].

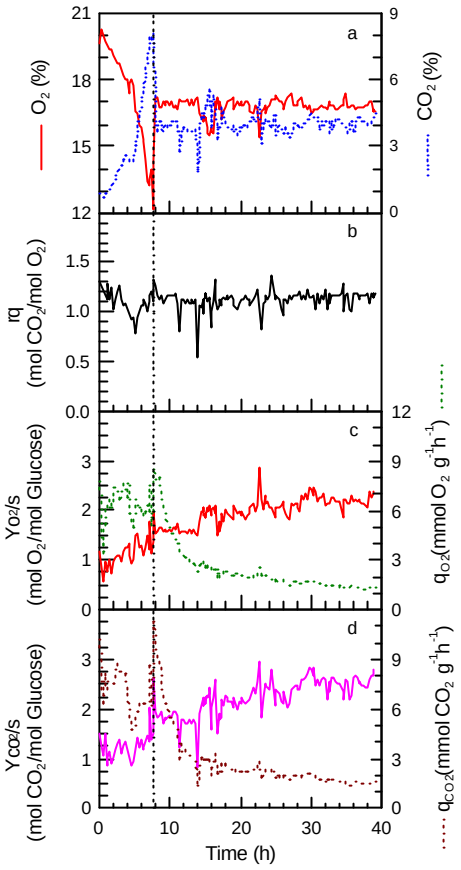


Figure 3.35: Respiration data during a large-scale fermentation with *E. coli* W3110 [ET01].

The on-line measurement during fed-batch cultivation of *E. coli* W3110 indicated that DOT and pH depend on the place in the reactor where the electrode are installed, which suggests reactor inhomogeneity (Fig. 3.34a,c). In this fermentation foaming was caused overtitration of NH_3 and hence the pH increased to 7.5 after 5 h of fermentation (Fig. 3.34c). Due to the limitation of power input, the stirrer speed only ranged from 50 rpm to 125 rpm (Fig. 3.34b). The DOT was regulated by raising the air flow to the reactor limit of 0.8 vvm. Therefore, an increased CO_2 concentration of 8 % in exhaust gas was observed during the exponential feed phase. After changing to constant feed, the concentration of exhaust O_2 and CO_2 remained approximately constant and the respiration quotient RQ was about 1.1 (Fig. 3.35a,b). This means that the amount of produced carbon dioxide per consumed glucose ($Y_{CO2/S}$) and the amount of consumed oxygen per consumed glucose ($Y_{O2/S}$) increased with time. Theoretically, the quotient $Y_{O2/S}$ is 6 for the complete oxidation of glucose. The quotient $Y_{O2/S}$ increased from 1.7 to 2.5 during the cultivation after constant feed start and indicated that more and more of the consumed glucose was used for maintenance energy.

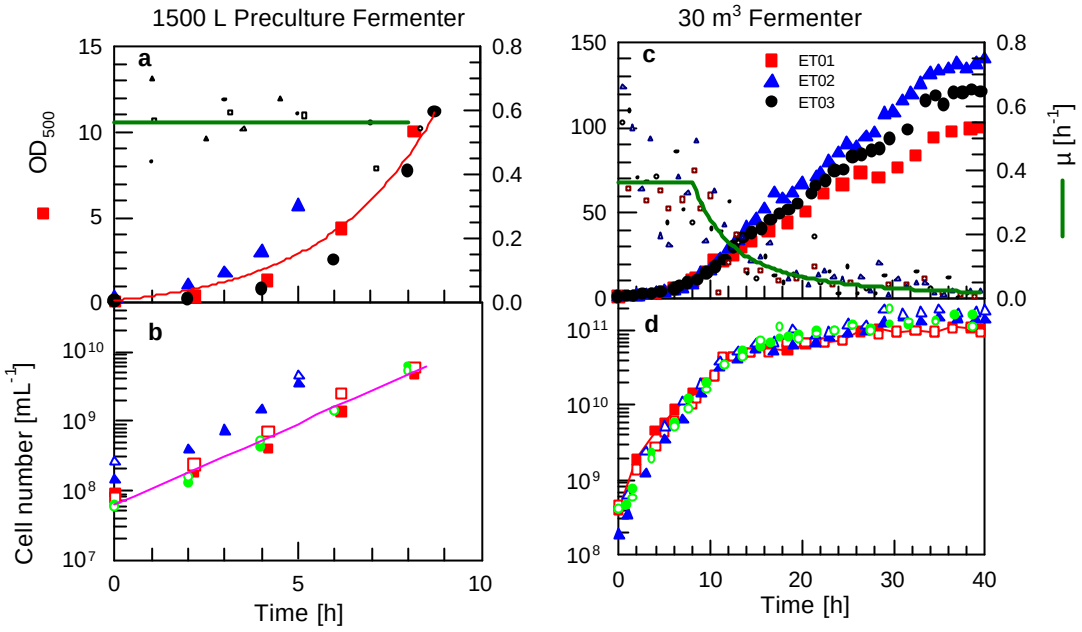
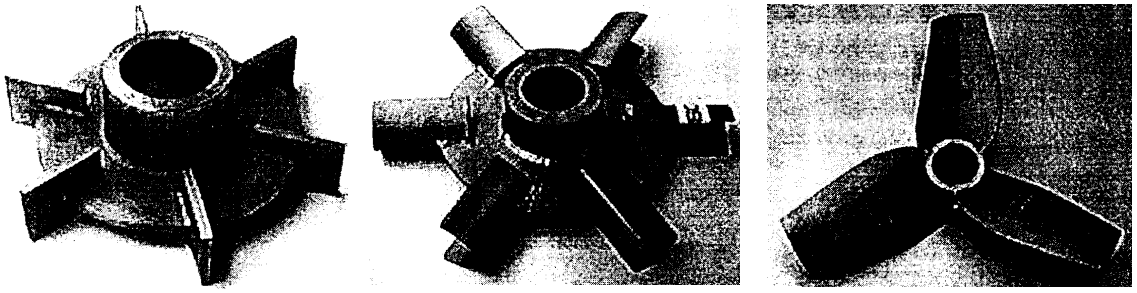


Figure 3.36: Comparison of three similar large-scale cultivations of *E. coli* W3110 [ET01 (■), ET02 (▲), ET03 (●)]. **a** and **b** show the pre-cultivations in the 1500 L bioreactor, whereas **c** and **d** show the fed-batch fermentations in the 30 m^3 bioreactor. **a**, **c**) OD_{500} (closed symbols) and specific growth rate μ (open symbols); **b**, **d**) colony forming units (open symbols), and total cell number (closed symbols).



(**a**) Rushton stirrer (radial pumping)

(**b**) Scaba 6SRGT (radial pumping)

(**c**) Scaba 4SHP (axial upward pumping)

Figure 3.37: Stirrers used in the large-scale cultivations (Vrábel et al., 1999b; for further information see Table 3.2).

A comparison of cell growth from three similar large-scale cultivations with different stirrer configuration (Fig. 3.36) is presented in Figure 3.37. The final values of these fermentations are summarized in Table 3.2. The data from the precultures in the 1500 liter scale indicated an exponential growth phase by optical density and colony forming units with a specific growth rate of 0.56 ± 0.05 h^{-1} (Fig. 3.36a,b). During preculture a OD_{500} of 1 corresponded to a cell number of 6.5×10^8 mL^{-1}. The growth in fermentations ET01-03 was very similar and no cell segregation into non-culturable cells was observed to the end of the cultivations (Fig. 3.36d). The optical cell density profile in the 30 m^3 scale indicates two distinct phases (Fig. 3.36c) according to exponential feed and constant feed phase. Within the first 10 hours cells grew with a constant specific growth rate of

$0.35 \pm 0.05\ h^{-1}$. Interestingly, a higher overall biomass yield was observed in the fermentations ET02 ad ET03 with the Scaba impeller configuration (Table 3.2).

Table 3.2: Comparison of the large-scale cultivations ET01-03 of *E. coli* W3110.

Fermentation		ET01	ET02	ET03
Dry cell weight	$g\ L^{-1}$	37.4	42.9	40.4
OD_{500}		106	140	121
Total cell number	$10^{11}\ mL^{-1}$	1.1	1.4	1.3
Colony forming units	$10^{11}\ mL^{-1}$	1.1	1.4	1.3
Final yield Yx/s	$g\ g^{-1}$	0.28	0.36	0.36
Final glucose	$mg\ L^{-1}$	47	200	276
Bottom impeller type		Rushton Φ= 700 mm (radial pumping) (hxb*=140x170 mm)	Scaba 6SRGT Φ= 1050 mm (radial pumping)	Scaba 6SRGT Φ= 1050 mm (radial pumping)
2nd, 3rd and 4th impeller		Rushton Φ= 700 mm (radial pumping) (hxb=140x170 mm)	Scaba 4SHP Φ= 1150 mm (upward pumping)	Scaba 4SHP Φ= 1150 mm (upward pumping)
Impeller spacing		1460 mm	1460 mm	1460 mm
Glucose feed point		H=6500 mm close to wall between baffles	H=6500 mm close to wall between baffles	H=2780 mm close to 2nd impeller tip
NH_3 feed point		above liquid surface	H=42000 mm close to 3rd impeller tip	H=42000 mm close to 3rd impeller tip

*: hxb = blade height x blade width

The results suggest that the mixing efficiency and residence time affected the cell growth and cell physiology during large-scale cultivation. Mixing in bioreactor depends on energy input, impeller type, reactor configuration and impeller geometry. Rushton turbines (Fig. 3.37a) were introduced in industry, mostly due to the good gas dispersion capabilities (Nienow, 1998). Compartmentalization and consequently strong axial flow barriers are associated with such radial flow impellers (Cronin et al., 1994). However, axial upward pumping Scaba impellers improved mixing compared to radial Rushton turbines because of the reduction of axial flow barriers by the different circulation flow patterns. Furthermore, the combination of multiple radial and axial Scaba impellers resulted not only in reduction of mixing time, but also in a lower loss of mechanical power, whereas gas hold-up at equal aeration and power input was not changed (Vrábel et al., 1999; for more information see project report).

The content of adenosine phosphates (AXP) was measured during fermentation ET03 (Fig. 3.38). A fluctuation in the energy charge was observed during the fermentation, which ranged between 0.9

and 0.4. The level of AMP was found to be between 0 and 3 mol g^{-1} DCW, ADP between 1 and 14 µmol g^{-1} DCW, and ATP between 1 and 10 µmol g^{-1} DCW.

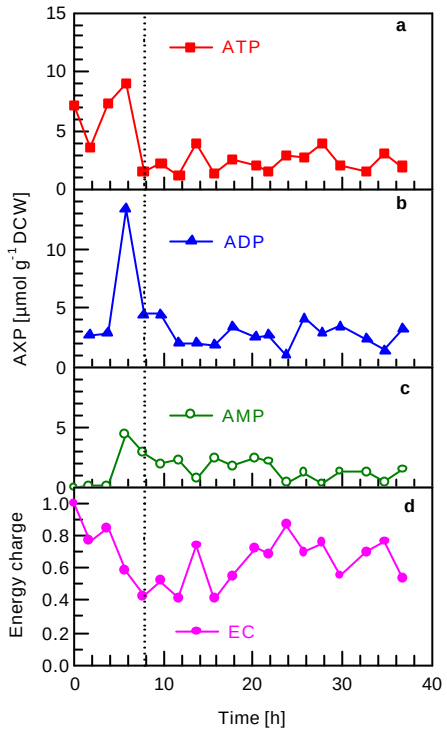


Figure 3.38: Intracellular AXP concentrations and energy charge during a large-scale glucose limited fed-batch fermentation [ET03] of wildtype *E. coli* W3110. The dotted line indicates the switch from exponential feed to constant feed.

3.4.2 Large-scale cultivations of recombinant *E. coli* W3110 pRIT44T2

Fig. 3.39 shows the growth characterization of *E. coli* W3110 pRIT44T2 in two 2 large-scale cultivations, which performed in the 12 m^3 stirred tank with Rushton and/or Scaba impeller configuration. The data from the preculture with 1200 liter scale show that the culture is exponentially growing with a specific growth rate of 0.48 ± 0.05 h^{-1} (Fig. 3.39a,b). Similarly to the large-scale cultivations of the wildtype W3110, during preculture an OD_{500} of 1 corresponded to a cell number of 6.5×10^8 mL^{-1}. Table 3.3 summarizes some experimental results and the stirrer configurations in the both experiments PU01-02. The biomass yield per glucose ($Y_{X/S}$) at induction point was determined to be 0.37 ± 0.01 g g^{-1}. After induction, the $Y_{X/S}$ decreased to 0.29 g g^{-1}. The product accumulation was much higher with the Scaba stirrer (PU02) and reached higher level of 59.8 mg g^{-1} DCW. Compare to this, only 38.6 mg ZZ protein per gram DCW were obtained in cultivation PU01.

The cell number was analyzed by microscopy and reached a maximal value of 6×10^{10} mL^{-1} shortly after induction with IAA (Fig. 3.39d). In both cultivations, no further increase of the total cell number was observed in 1-2 hours after induction. Replica plating data indicated no plasmid loss after induction during both cultivations. However, a significant difference was detected between both fermentations concerning cell culturability (Fig. 3.39d). In fermentation PU01 with the Rushton stirrer

set, non-dividing cell started to occur in 1 h after induction. The part of the population, which lost its colony forming ability was about 30 % of the total cells to the end of the experiment. In contrast to this, no cell segregation was found in the fermentation PU02 with the Scaba stirrer set. Almost all cells maintained their ability to form colonies on plates. The large-scale experiments of *E. coli* W3110 pRIT44T2 in 12 m^3 showed almost no plasmid amplification (Fig. 3.40).

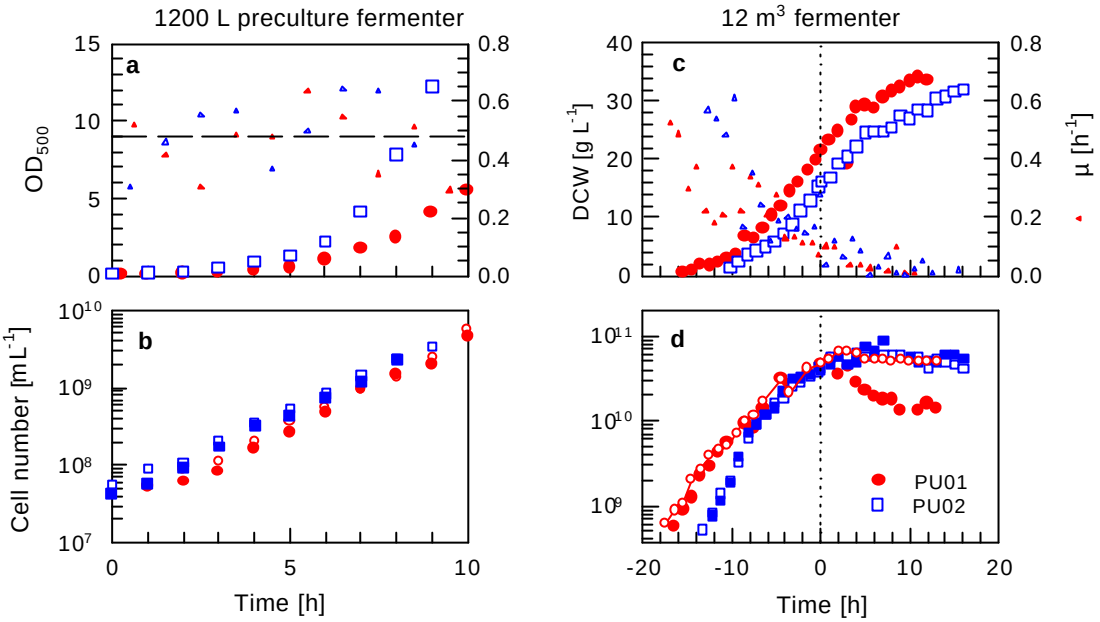


Figure 3.39: Cell growth, total cell number and colony forming units during two similar cultivations of *E. coli* W3110 pRIT44T2 with induction by IAA [PU01 (●), PU02 (□)]. **a**, **c**) cell growth; **b**, **d**) colony forming units(●,■) and total cell number(○,□) during pre-cultivations in a 1200 L bioreactor (a, b) and fed-batch fermentations in a 12 m^3 bioreactor (c, d). Dotted line mark the time points of the induction with IAA.

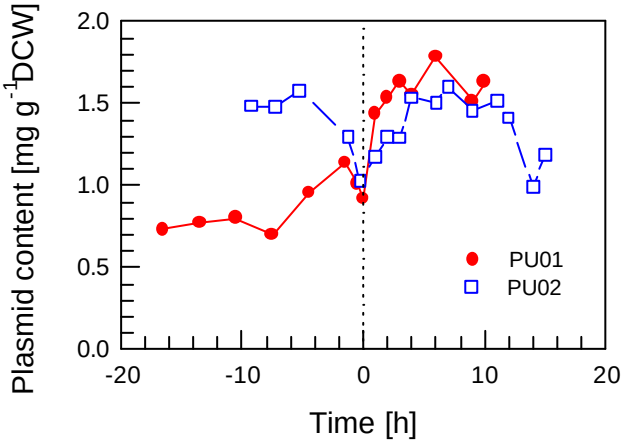


Figure 3.40: Plasmid content during large-scale fed-batch cultivations of *E. coli* W3110 pRIT44T2 with induction by IAA. PU01 (Rushton stirrer set) with filled symbol; PU02 (Scaba stirrer set) with open symbol. Dotted line mark the time points of the induction with IAA.

Table 3.3: Comparison of the 12 m^3 scale cultivations PU01-02 of *E. coli* W3110 pRIT44T2.

Fermentation		PU01	PU02
Dry cell weight (DCW)	g L^{-1}	33.5	32.1
OD_{500}		72	135
Total cell number	10^{10} mL^{-1}	5.0	5.0
Colony forming units (cfu)	10^{10} mL^{-1}	1.3	5.0
Plasmid stability	%	100	100
Cell length (Hewitt et al, 1998)	µm	2.8	3.7
ZZ (maximal/final value)	mg g^{-1}	38.6 / 29.9	59.6 / 21.8
yield Yx/s at induction / final	g g^{-1}	0.37 / 0.29	0.38 / 0.29
Bottom impeller type		Rushton Φ= 700 mm (radial pumping) (hxb=110x210 mm)	Scaba 6SRGT Φ= 950 mm (radial pumping)
2nd and 3rd impeller		Rushton Φ= 700 mm (radial pumping) (hxb=110x210 mm)	Scaba 3SHP Φ= 1050 mm (upwards pumping)
Impeller spacing		850 mm	850 mm
Glucose feed point		H=1700 mm, 400 mm from the wall, close to 2nd impeller tip	H=1700 mm, 400 mm from the wall, close to 2nd impeller tip
NH_3 feed point		in the inlet air via the sparger	in the inlet air via the sparger

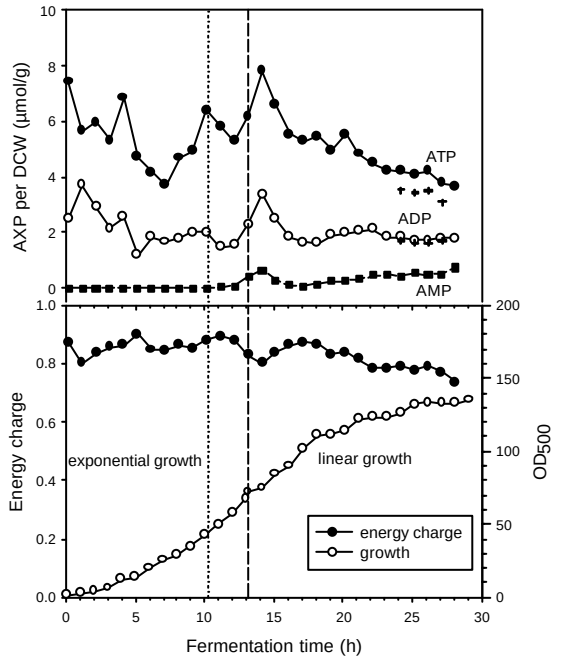


Figure 3.41: Intracellular AXP concentrations and energy charge during a glucose limited fed-batch fermentation of recombinant *E. coli* W3110 pRIT44T2. Dotted line marks the time points of start of constant feed and interrupted line marks the induction with IAA, respectively. The points marked with crosses correspond to values corrected for extracellular AXP. (Data from Meyer et al, 1999)

From the data of nucleotide analysis of PU02 by Meyer and coworkers (1999), the ATP concentration ranged from 8 µmol g^{-1}DCW to 4 µmol g^{-1}DCW before induction where the fluctuate was suggested to be caused by the sampling performance and the inhomogeneity in the bioreactor (Fig. 3.41a). This phenomenon was stronger in the larger 30 m^3 fermenter of no-recombinant *E.coli*

W3110 (see Fig. 3.38). A slight increase in ATP and ADP level was observed direct after induction but just lasted for one hour, which correlates to the data obtained in the α-glucosidase process and might be caused by an increased cellular metabolism due to overexpression of ZZ. Then ATP and ADP levels decreased sharply within 2 hours. Afterwards, a slight decrease in ATP level was observed towards the end of the fermentation, whereas the ADP concentration was constantly low (Fig. 3.41). The AMP level remained constant before induction and increased slightly towards the end after induction. The energy charge was very stable up to induction (about 0.85) and decreased slightly towards the end of the fermentation (0.75). Furthermore, an increase of the extracellular concentration of the adenosinphosphates was detected after induction (Meyer et al, 1999).

The results obtained with the recombinant ZZ production system indicated that the formation of high levels of recombinant product had little effect on the cell growth, although the cell segregation into viable but non-culturable cells was found in the cultivation PU01 with the Rushton stirrer set. Furthermore, the plasmid pRIT44T2 is stabily maintained and showed no amplification after induction of the ZZ protein. Similar to the CRIMI system, no up-growth of plasmid-free cells appeared.

3.4.3 Cell lysis and cAMP level in large-scale processes

Cell lysis in large-scale processes. To investigate the cell lysis in large-scale cultivation, the amount of extracellular protein content was determined and represented in Fig. 3.42. Although the final specific extracellular protein content in media is about 0.01 g g^{-1}DCW in all cultures, a substantial difference in accumulation of extracellular protein during first ten hours was found between the two systems and even the cultivation of the same system, such as cultivation ET02 and ET03. The cell lysis is higher in the case of *E. coli* W3110 in 30 m^3 scale than the recombinant system for overproduction of ZZ in the 12 m^3 scale. Within the first 10 hours in cultivation ET03, the extracellular protein was accumulated exponentially with a rate of 0.34 h^{-1}, which was similar to the cell growth. If assuming that the cellular protein content was 50 % of the dry cells, cell lysis would account for about 5 % in cultivation ET03. After 20 h in cultivation ET02 and 15 h in cultivation ET03 no more increasing of extracellular protein was detected, and the specific content decreased to 0.08 g g^{-1} DCW. It is obvious that the cell lysis is higher in large-scale cultivation than in the laboratory scale. Furthermore, the analysis of the extracellular protein content showed a slight increased cell lysis after induction. The extracellular protein per cell units decreased during

exponential phase and stayed constant after constant feeding, whereas it slightly increased after induction with IAA.

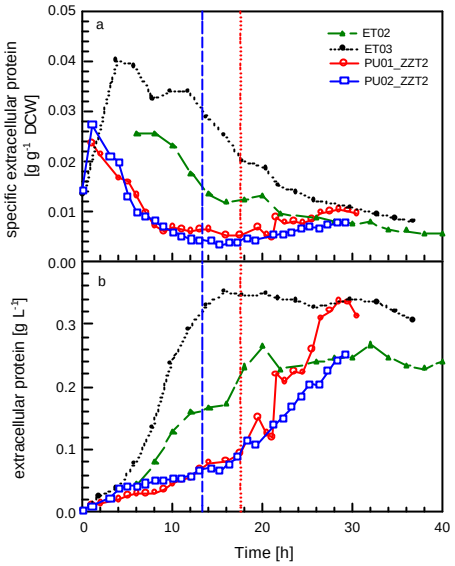


Figure 3.42: Extracellular protein content during cultivations of *E. colli* W3110 with and without pRIT44T2 in large-scale 12 m^3 and 30 m^3 fermenter. The dotted line (for PU01) and interrupted line (for PU02) mark the time points of the induction with IAA respectively.

Extracellular cAMP level in large-scale processes. Fig. 3.43 shows the extracellular cAMP content during cultivations of *E. colli* W3110 with and without pRIT44T2 in large-scale 12 m^3 and 30 m^3 fermenters. The data show a good reproducibility between different fermentations despite the change in the stirrer geometry. A clear difference between the two systems, with and without overproduction of ZZ, was found in the accumulation of extracellular cAMP in the cultivation medium. The process with wildtype strain showed a higher cAMP formation rate than the process with the recombinant strain.

The extracellular cAMP concentration was also measured in corresponding laboratory scale cultivations which were performed with a similar profile (see Fig. 3.43). The wildtype *E. coli* W3110 strain was cultivated to a cellular density of 48 g dry cell weight per liter in a 10 L fermenter by the group of S.-O. Enfors (KTH, Stockholm) within the EU-"scale-up" project (Fig. 3.44a). The fermentation was slightly changed in relation to the large-scale culture as it started with a short batch (for 4 hours), where cells grew at their maximum specific growth rate of 0.58 h^{-1}. Afterwards the feed was started with an exponential feed allowing a constant specific growth rate of 0.26 h^{-1} for 6 h, and finally a constant feed was applied. The specific cAMP content reached a level of 4 µmol g^{-1} DCW after the depletion of glucose and showed then a slightly decreasing tendency, similar to the corresponding large-scale cultivation.

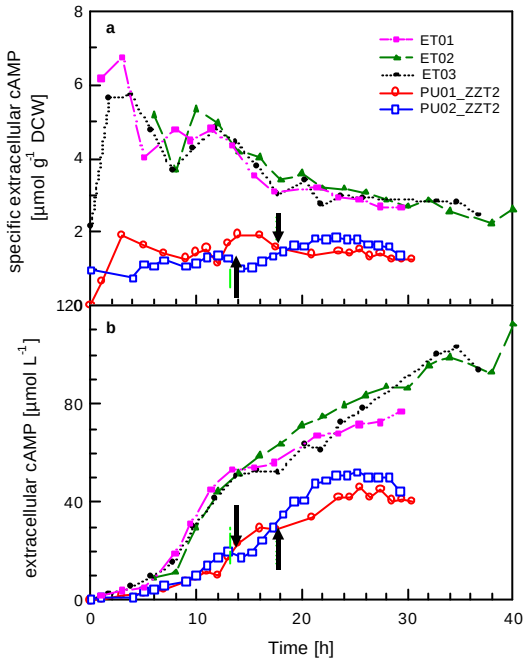


Figure 3.43: Extracellular cAMP content during cultivations of *E. colli* W3110 with and without pRIT44T2 in large-scale 12 m^3 and 30 m^3 fermenter. Arrow shows the time point of induction. Exponential feeding started at 1 h after fermentation start, whereas constant feeding started at 10 ± 1 h corresponding a OD_{500} about 10.

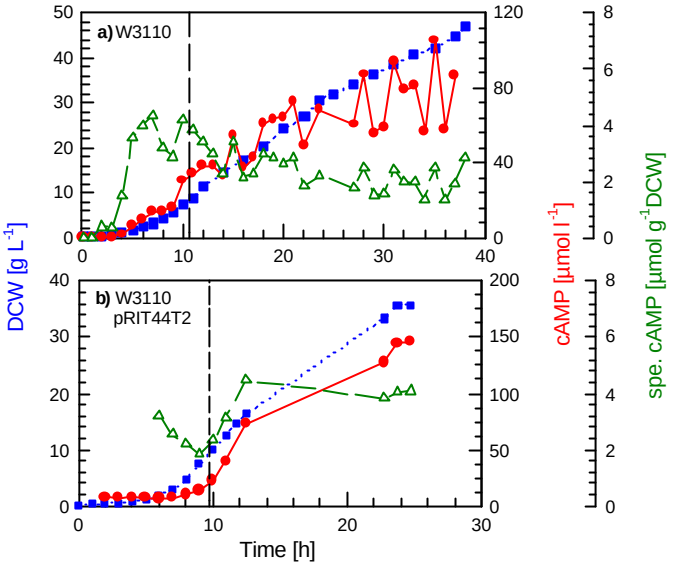


Figure 3.44: Cell growth (■) and extracellular cAMP concentration (● µmol L^{-1}; Δ µmol g^{-1} DCW) in small scale glucose limited fed-batch cultivations of *E. coli* W3110. **a**) *E. coli* W3110 in 10 L fermenter, **b**) recombinant W3110 pRIT44T2 without induction in 5 L Biostat ED. The interrupted line marks the time point of change from exponential to constant feed (**a**) or from batch to constant feed (**b**). Medium samples in (**a**) were treated with 0.135 M perchloric acid at the time of sampling, to stop all metabolic activity.

The recombinant cultivation of *E. coli* W3110 pRIT44T2 was performed in 5 L Biostat ED fermenter (Fig. 3.44b). The level of cAMP was higher in batch phase and accumulated exponentially to 22 µM with a rate of 0.40 h^{-1} till start of constant feed. After total consumption of initial glucose the cAMP concentration has increased linearly with a rate of 20.2 µmol L^{-1} h^{-1} for 3 hours and then stayed at a high level of 150 µM in corresponding to 4 µmol g^{-1} DCW.

If the cAMP data from the large-scale fermentation of W3110 (Fig. 3.43a) are set in correlation to the data obtained in the small-scale experiment, which was performed with the same feeding profile (see Fig. 3.44a), one could conclude that the different extracellular cAMP level (expressed in µmol g^{-1} DCW) is caused by the different scale. Although further experiments would be validated this effect, it might be that the oscillations in the large reactor caused the higher cAMP release. In contrast to this wildtype strain, in the recombinant system (W3110 pRIT44T2) no higher cAMP level in the cultivation medium could be detected, when this strain was cultivated at a large-scale (compare Fig. 3.43b and Fig.3.44b).

3.5 Influence of glucose oscillations on the α-glucosidase process by using a scale-down technique

One of the methods to study large-scale effects in a laboratory bioreactor is to use an intermittent feed protocol. This method in comparison to other methods, such as a two compartment reactor, showed an increased response because all cells are exposed synchronously to the stressing conditions (Neubauer et al., 1995a,b). This chapter was focused on the main model system for overproduction of the α-glucosidase in *E. coli* fed-batch cultivation by using two different feed protocols based on the results of Neubauer (1995a,b). The first one was performed with a 4 min cycle of 2 min feeding and 2 min shut off of the feed flow, whereas the second profile was a shorter cycle of only one minute with 30 sec feeding and a pump-off phase of 30 sec since earlier studies suggested an increased stress situation in a shorter cycle (Neubauer et al, 1995a). Although this investigation was performed in a small scale reactor situation by an intermittent feed protocol and would have to be extended in the future to analyze in large-scale processes, this study provides useful information on which kind of responses should be looked at in the large scale process.

3.5.1 Effect of glucose oscillations on cell growth and α-glucosidase formation

A comparison of the three different types of cultivation, continuous feeding and two intermittent feeding with different feed cycles, shows no significant difference in growth during the phase from the feed start to the point of IPTG addition in both systems of *E. coli* RB791 pKK177glucC with and without pUBS250 (Fig. 3.45). During the cultivations without pUBS520 (Fig. 3.45a-c), cell growth was inhibited from 3 h after induction at a DCW of about 10 gL^{-1} independent of the feed mode. But in the cultivation with short cycles (1 min), the cell restarted to grow till a DCW of about 20 $g\,L^{-1}$. Furthermore, a decreased formation of α-glucosidase was observed in the culture with long term cycles (4 min), whereas an increased accumulation of α-glucosidase was found in culture with short cycles (1 min). The results indicated that the length of cycle interval played an important role and affected the cell growth and product formation (see Table 3.4).

The similar phenomenon was also found in the cultures with pUBS520 (Fig. 3.45d-f). Growth was inhibited in all cultures after induction and the corresponding accumulation of α-glucosidase showed approximately the same specific and overall rates in all cultures. However, during prolonged cultivation the amount of α-glucosidase decreased to a very low level in the culture with long term cycles (4 min), but the amount was similar to the control culture when short cycles (1 min) were applied (Fig. 3.45f). As observed in the control culture, the majority of the product α-glucosidase

was found as inclusion bodies. The decrease in product concentration per cell unit in the cultivation with long cycle (4 min) cannot be explained by the up-growth of plasmid-free cells, since the total concentration of α-glucosidase in the culture also decreased (data not shown). I assume that the higher level of stress, which was earlier detected by the response of the alarmone ppGpp (Neubauer et al., 1995a), could be connected to expression of proteolytic factors, such as Lon and ClpP.

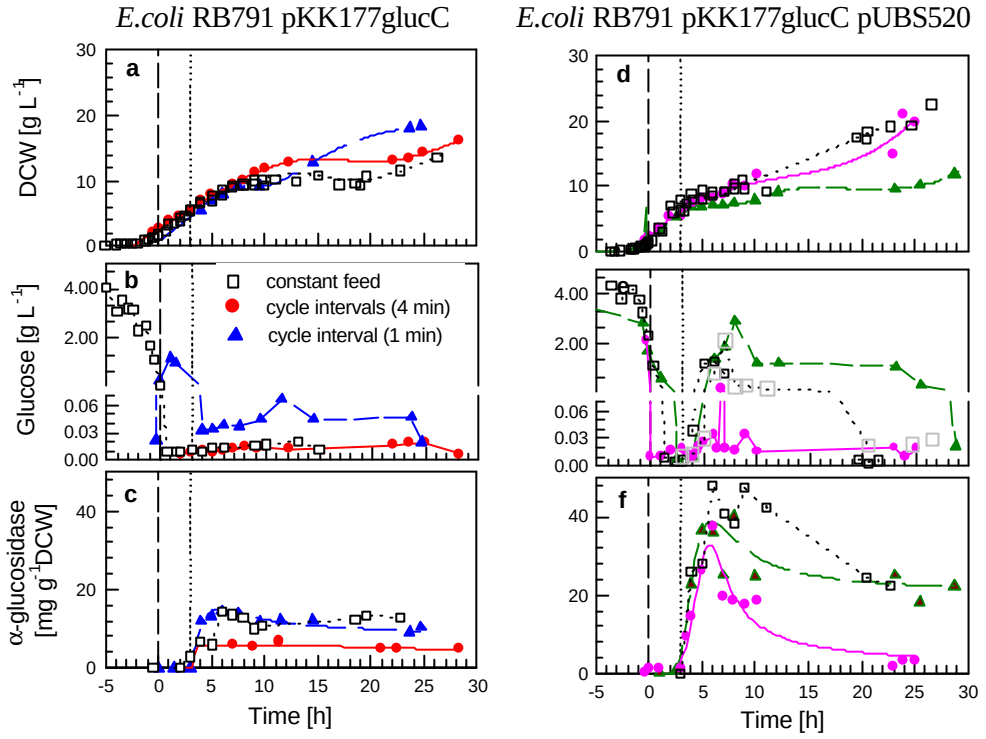


Figure 3.45: Comparison of the effect of glucose oscillations on cell growth (**a**, **d**), substrate consumption (**b**, **e**) and total product formation (**c**, **f**) during fed-batch cultures of *E. coli* RB791 pKK177glucC (**a-c**) and *E. coli* RB791 pKK177glucC pUBS520 (**d-f**) with constant feed (□) and with intermittent feed. The cycle intervals were 4 min (●) or 1 min (▲).

Table 3.4: Comparison of cultivation of *E. coli* RB791 pKK177glucC with or without pUBS520 with constant feed and intermittent feed.

Feed mode [constant or intermittent feed]	α-glucosidase [mg g^{-1} DCW]		plasmid-free cells [% total population]	
	3 h a.i.[a]	20 h a.i.	3 h a.i.	20 h a.i. /12h a.i.
RB791 pKK177glucC pUBS520				
constant feed	37	30	2	72
cycle 1 min	38	24	1	16
cycle 4 min	37	6	2.5	60
RB791 pKK177glucC				
constant feed	10	9	10	10 / 3.4
cycle 1 min	14	10	0.3	2.7 / 0.1
cycle 4 min	6	4.6	15	6.7 / 6

[a]: a.i. ≙ after induction.

Difference in glucose consumption and acetate formation was observed between the two feed protocol. Glucose was accumulated to a maximum level of 2.6 g L^{-1} after induction if intermittent feed with short term cycles (1 min) was applied, similar to the glucose profile for the control fermentation (Fig. 3.45e). But glucose concentration showed no increase to the g per liter level in the case of long cycles. Glucose was only non-limiting for a very short time, which can also be concluded from the DOT and outgas data at 2.5 h to three hours after induction (see Fig. 3.49c,e). Correspondingly, the acetate level only slightly increased for a relative short time to about 0.3 g L^{-1} (Fig. 3.46).

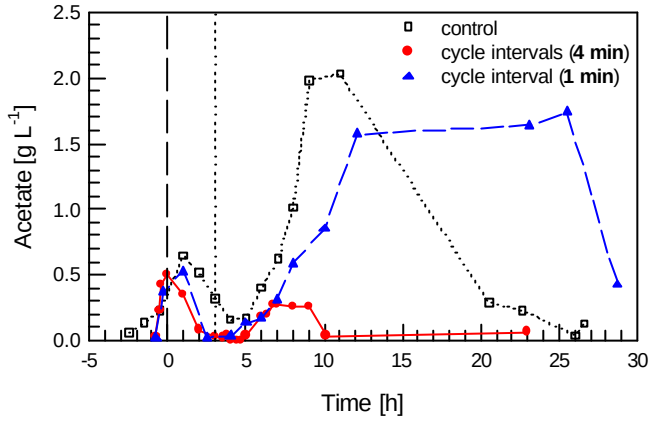


Figure 3.46: Comparison of the effect of glucose oscillations on acetate formation and re-consumption during fed-batch cultures of *E. coli* RB791 pKK177glucC pUBS520 with constant feed (□) and with intermittent feed. The cycle intervals were 4 min (●) or 1 min (▲).

The results from both systems with and without pUBS520 indicate that glucose oscillations influence the cell growth and the product stability. The glucose uptake capacity was strongly inhibited in the cultures if continuous feed and short cycle (1 min) were performed. For a more comprehensive understanding, the question about the effect of glucose oscillation on cell segregation and up-growth of plasmid-free cells will be discussed in the following section.

3.5.2 Effect of controlled glucose oscillations on cell segregation and maintenance

Plating data during the cultivations of *E. coli* RB791 pkk177glucC pUBS520 showed that the cells which were stressed by repeated long term (2 min) glucose starvation had a lower decrease of the colony forming ability for about three hours after induction compared to the control (Figs. 3.47a, b). The final viability after 25 hours of cultivation was approximately five to ten times higher than in the control culture with continuous feed. However, the long cycle culture showed about the same proportion of plasmid-free cells as the control. The final fraction of this subpopulation was 60 % at the end of the fermentation (Fig. 3.47b).

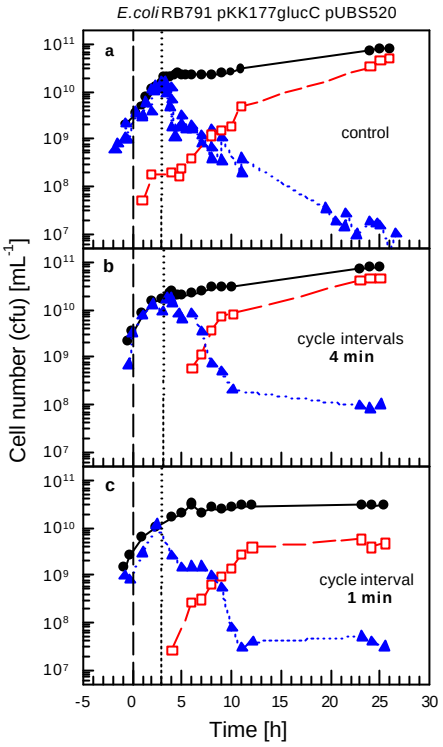


Figure 3.47: Comparison of total cell number (●), colony forming units of plasmid-containing cell (▲) and cfu of plasmid-free cell (□) during fed-batch cultivations of *E. coli* RB791 pKK177glucC pUBS520 with constant feed (**a**, as control) and with intermittent feed with cycle intervals of 4 mins (**b**) or cycle interval of 1 min (**c**).

There was no significant up-growth of a plasmid-free population when glucose was intermittently fed in one minute cycles (Fig. 3.47c). Although α-glucosidase producing cells lost their colony forming ability by approximately the same rate as the cells in the control culture (Figs. 3.47a and c), which resulted in glucose becoming non-limiting and reaching 3 g L^{-1}, the specific growth rate (μ_{max}) of the plasmid-free population was only 0.46 h^{-1} compared to 0.61 ± 0.1 h^{-1} for the control. The explanation for this lower μ_{max} value can be drawn from the outgas data. When such short cycles were performed, the carbon dioxide production rate was much higher, which was especially obvious from the yield coefficient for CO_2 on glucose ($Y_{CO2/S}$), which was 2 to 2.5 mol mol^{-1} at the point of induction in the control and the long cycle cultures, but was 4mol mol^{-1} for the culture with one minute feed cycles (Fig. 3.48c). The higher CO_2 production increased the maintenance coefficient and caused a reduction of the maximum specific growth rate.

The similar effects: inhibition of up-growth of the culture by plasmid-free cells and higher CO_2 production in the same range, were also observed in cultures where the feed was pulsed in the same one minute cycles, but where the strain did not contain the plasmid pUBS520 (data not shown).

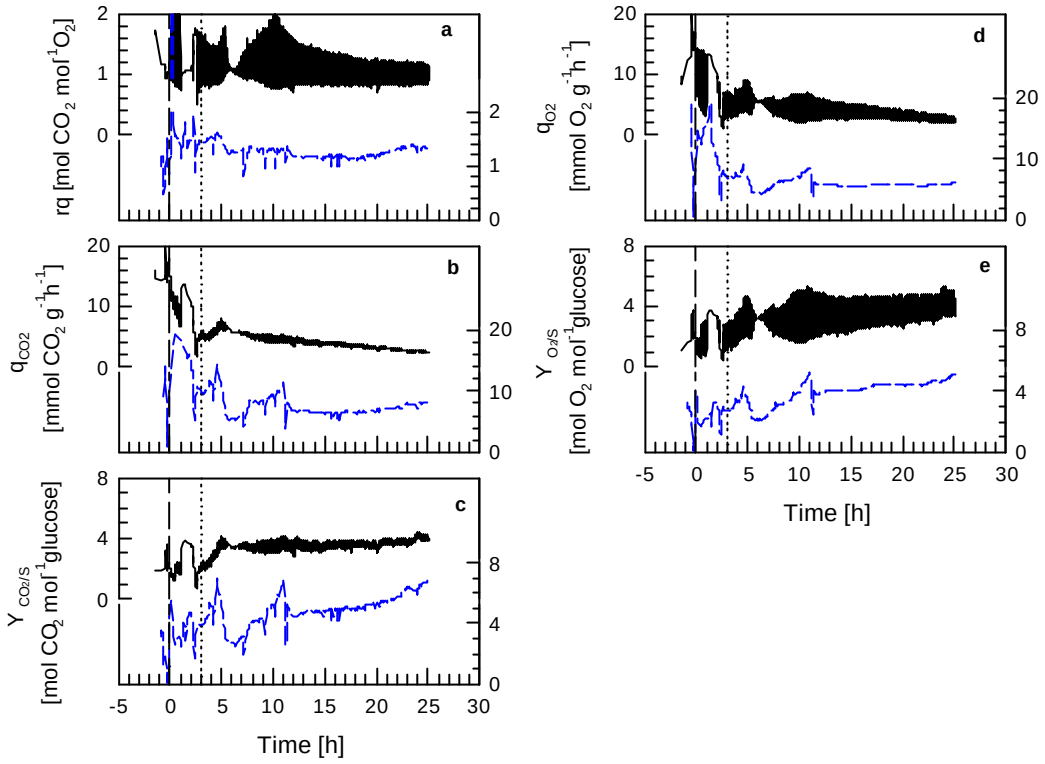


Figure 3.48: Effect of glucose oscillations on the respiratory quotient (RQ, **a**), carbondioxid evaluation rate (q_{CO2}, **b**), oxygen uptake rate (q_{O2}, **d**), yield of carbondioxid and oxygen per glucose ($Y_{CO2/S}$, **c**; $Y_{O2/S}$, **e**) during fed-batch cultures of *E. coli* RB791 pKK177glucC pUBS520 with intermittent feed. The cycles were 4 min (——, upper level) and 1 min (----, lower level). Induction was performed at 3 h after feed start by 1 mM IPTG.

In contrast to CO_2 formation profiles, no significant difference could be detected in the parameters describing the respiratory activity. In both cultures with oscillatory glucose feed the actual respiratory activity was following the pulses as it is obvious from the DOT data (Fig. 3.49) and the outgas analysis (Fig. 3.48). The specific rate of oxygen uptake (q_{O2}) and the yield coefficient $Y_{O2/S}$ were not significantly different from the control values when compared at the point of induction (q_{O2} = 6 mmol O_2 g^{-1} h^{-1}; $Y_{O2/S}$ = 2.5 mol mol^{-1}). Interestingly, the phases where glucose is not limiting, are directly inferred from the DOT signal (Fig. 3.49) due to the inhibition of glucose uptake following induction of α-glucosidase. This response can be used to control feeding to prevent glucose and acetate accumulation. Similarly, the up-growth of plasmid-free cells can be decreased by decreasing the feed rate, which could be easily done based on the DOT response in the case of the 4 min oscillation cycle.

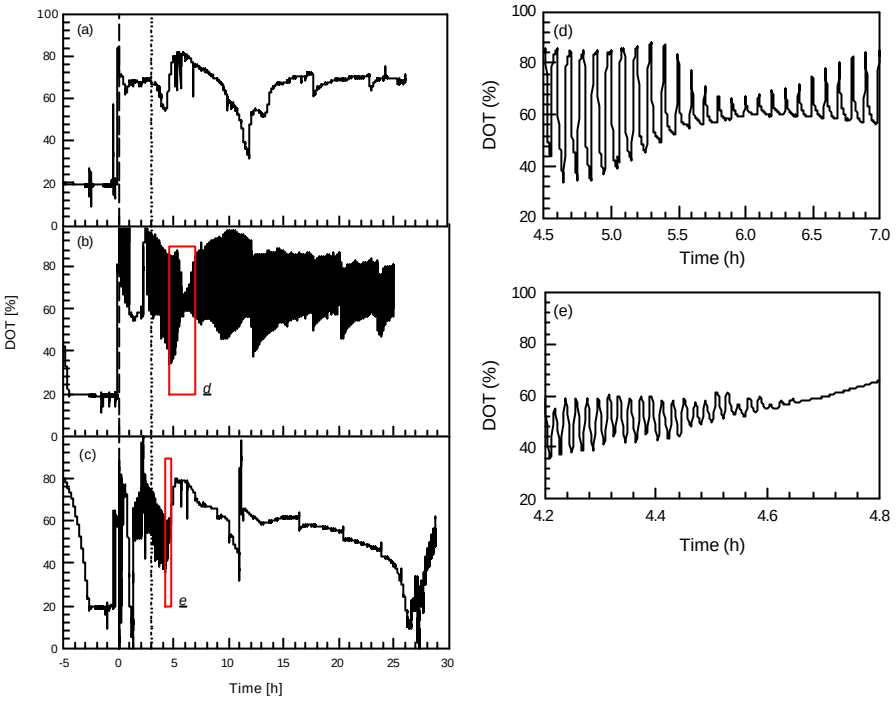


Figure 3.49: DOT in response to glucose oscillations during different cultivation of *E. coli* RB791 pKK177glucC pUBS520 with constant feed (**a**) and intermittent feed cycles of 4 min (**b**; **c**) or 1 min (**d**; **e**).

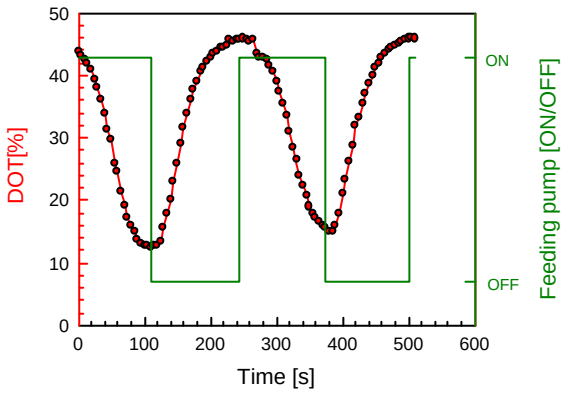


Figure 3.50: The dissolved oxygen tension (DOT) in response to the intermittent feeding of glucose, at the time of 6 h after feeding start during a cultivation of *E. coli* RB791 pKK177glucC with intermittent feed (cycle intervals of 4 min).

Fig 3.49 shows the on-line data of dissolved oxygen tension in response to the intermittent feeding of glucose. The oscillation of the DOT signal can be observed during cultivations with intermittent feeding. Fig. 3.50 shows the DOT response in two cycles with feed pump on and off with interval of 2 min. When the pump started to feed glucose solution to the reactor, the cells increased respiration and the DOT went down. After 2 min when the pump stopped, the cells were exposed to glucose-starvation, and decreased respiration caused an increase of the DOT-value. During repeated start

and stop of the glucose feed, the DOT was always oscillating. This oscillation in the outlet gas concentration of carbon dioxide and oxygen can been also observed.

3.5.3 Effect of controlled glucose oscillations on cell lysis and cellular responses

The analysis of the extracellular protein content in the cultivation medium was used to investigate whether oscillations affect the lysis of cells besides the influence on product stability and maintenance. As clearly shown in Fig. 3.51, the concentration of extracellular protein was low in all cases. A maximum lysis rate of 16 mg L^{-1} h^{-1} was determined during the early fed-batch phase, which was higher than the lysis rate after induction. However, Fig. 3.51 also showed that a slightly higher amount of cell lysis was found when fast oscillations were applied. The extracellular protein was lowest in fermentations with longer feeding interval. This seems to support the previous conclusion that the longer cycles of oscillatory starvation allow the cells to induce the response to starvation. Although a comprehensive analysis of protein expression patterns was not performed, the suggestion that the results can be explained by cell adaptation to stress, is supported by data obtained from immunoblot-analysis of the general starvation sigma factor σ^S, which was higher at the point of induction in cultures with oscillations than in control experiments (Fig. 3.52).

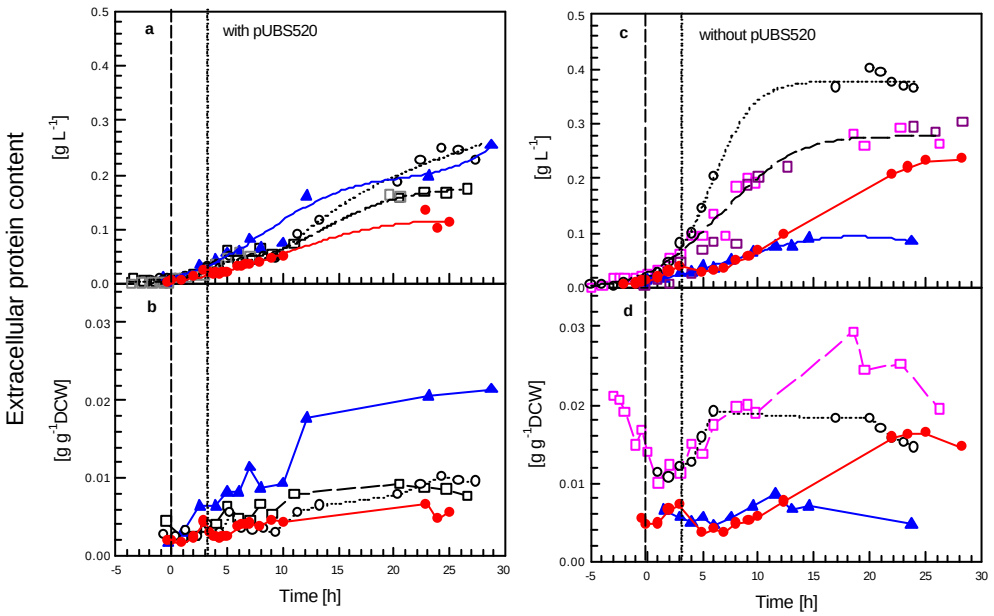


Figure 3.51: Comparison of extracellular protein content during different fed-batch fermentations of *E. coli* RB791 pKK177glucC pUBS520 (**a-b**) or *E. coli* RB791 pKK177glucC (**c-d**) with constant feeding without induction (O) and with induction (□), and intermittent feeding with cycles of 4 min (●) and 1 min (▲).

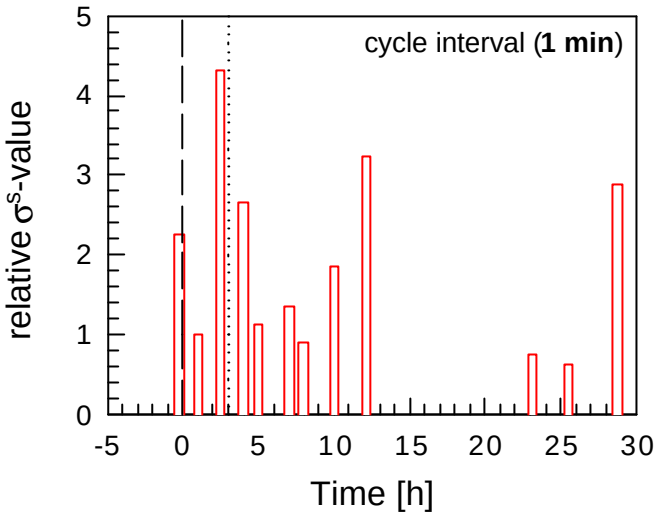


Figure 3.52: Concentration of σ^S during the cultivation of *E. coli* RB791 pKK177GlucC pUBS520 with intermittent feeding (cycle interval of 1 min). The interrupted line represents the time of feeding start and the dotted line indicates addition of IPTG.

The analysis of the plasmid content showed no significant influence of controlled glucose oscillations after induction (Fig. 3.53), although the plasmid content was slightly higher and stayed at this high value longer than during the control cultivation. In all three cases of *E. coli* RB791 pKK177glucC, the plasmid copy number increased by a rate of 133 $\mu g\, g^{-1}\, h^{-1}$ within 7 h after induction. Then the plasmid content stayed almost at the same level up to the end of the cultivation (Fig. 3.53b). Interestingly, a slightly increased plasmid level was found directly after the shift from the batch-phase to the feed phase in the cultivations with intermittent addition of the feed solution. We conclude, that repeated short time starvation provokes a higher plasmid content.

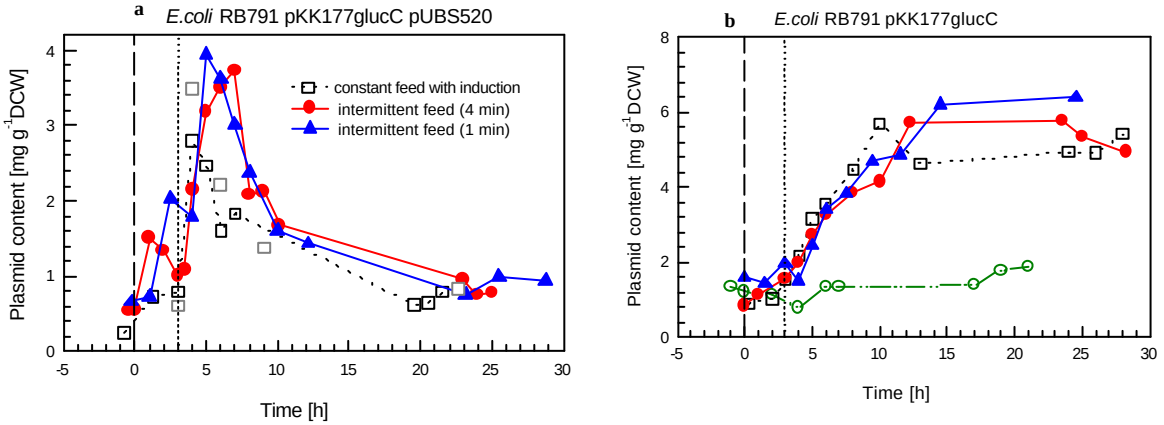


Figure 3.53: Effect of glucose oscillations on plasmid content during cultivations of *E. coli* RB791 pKK177glucC with or without pUBS520 with constant feeding (without induction (○) and with induction (□)), or intermittent feeding with cycle intervals of 4 min (●) and 1 min (▲).

Furthermore, the effect of glucose oscillations on ppGpp level during cultivations of *E. coli* RB791 pKK177glucC is presented in the Fig. 3.54. The ppGpp concentration in the three cultures was always lower after induction than in the fermentation without induction. By the shift from batch to fed-batch a drastic transient increase of ppGpp to approximate 0.5 µmol g^{-1} was found in the case of intermittent feed with longer cycle (4 min, Fig. 3.54). However, the same increase can be assumed for the other cultures since it has been shown that the analyzed value more depended on the time due to the fast overshoot response of ppGpp. In all oscillatory cultures ppGpp decreased after induction to the detection limit. Interestingly, this decrease appeared late in the culture with 4 min feed cycles, which was again a sign that the cells might be better adapted to stress in this case. The increase of ppGpp at 20 h after induction reflected the response to energy/carbon starvation of the upgrowing of plasmid-free cells. In this case again, the measured level of ppGpp depended on sampling time, whether pump was running or stopped, because the ppGpp content was considerably changing between 0.1 µmol g^{-1} and 0.7 µmol g^{-1} within a feeding cycle of 4 min as earlier shown by Neubauer et al. (1995b).

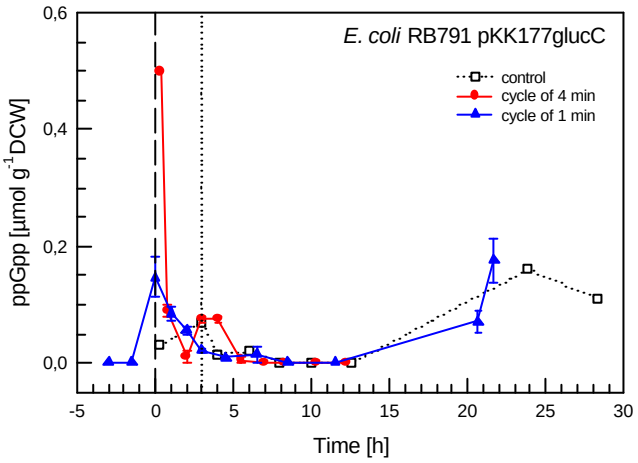


Figure 3.54: Effect of glucose oscillations on ppGpp level during cultivations of *E. coli* RB791 pKK177glucC with constant feeding (□), and intermittent feeding with cycles of 4 min (●) and 1 min (▲). All cultures were induced by 1 mM IPTG at 3 h after feeding start.

In summary, three main differences were found between cultures with constant feeding and intermittent feeding in the case of *E. coli* RB791 pKK177glucC without pUBS520: (1) Plasmid-free segregants grew up after induction if the constant feeding mode was applied and were detected from 3 h after induction. This plasmid-free population contributed to approximately 20% of the total population at 14 h after induction. The up-growth of plasmid-free cells was much lower in cultures with intermittent feeding. (2) A major cell population lost its ability to divide after induction independently of the strength of product expression. Furthermore, the number of cells, which lost the

ability for cell division, was lower in the case of intermittent feeding. (3) The specific and total concentration of extracellular protein was significantly lower in cultures where an intermittent feeding mode was applied.

The results from the strong expression system with pUBS520 indicated that glucose oscillations influenced the product stability and the up-growth of plasmid-free cells. Although after induction the glucose uptake capacity was inhibited in all cultures performed, the up-growth of the culture by plasmid-free cells was strongly inhibited by short oscillations. On the other hand, plasmid-free cells grew up when constant feeding or long cycles were applied. This behavior could be explained by an increased production of carbon dioxide from glucose during the short cycles. The corresponding $Y_{CO2/S}$ was $4\,mol\,mol^{-1}$ for short cycles but $2.5\,mol\,mol^{-1}$ for constant glucose feeding and long cycles. In connection to product formation the initial α-glucosidase accumulation was the same in all cultures, but the stability of the product was significantly lower in the cultivation with long cycles, possibly because of a higher level of general stress response.

4 Discussion

The strong overexpression of recombinant genes often results in a growth inhibition of the culture following induction. Undoubtedly, this growth inhibition results from major disturbances of the cellular functions of the host cell by the intensive formation of the recombinant product. A number of studies (for review see Bailey et al., 1993; Kurland et al., 1996) describe the possible interactions of the recombinant DNA or the subsequently formed proteins with the host cell metabolism, however, a thorough investigation is missing. Therefore, it was the major aim of this study to analyze comprehensively the change of the cells physiological state when a recombinant protein is induced to high levels for one recombinant model system. The aim of this chapter is to discuss these results in connection to the cytoplasmic expression of recombinant genes in general and to indicate in some points, by which factors differences could be caused.

4.1 Influence of recombinant gene overexpression on cell growth

Growth inhibition was found in the case of the α-glucosidase production independently of the coexpression of the argU-tRNA, which limits the synthesis of α-glucosidase. However, the growth inhibitory effect was more stronger in cultivations with the strain overexpressing the argU gene, although without induction of the α-glucosidase, coexpression of argU had no negative effect on the growth. Rather we even detected a positive effect on the specific growth rate of the culture with a value of 0.79 h^{-1} (μ_{max}) versus 0.68 h^{-1} (with pUBS520 versus without pUBS520). In the host with a higher content of argU-tRNA cell growth stopped within two hours after induction, which was connected to an increase of the levels of glucose and acetate in the growth medium. At the same time the cells lost their colony forming ability by an initial rate of 0.74 h^{-1} (k_d) resulting in a decrease of ability for reproduction by an order of magnitude of three within the time of cultivation. Although the loss of the ability for reproduction is dramatic, this process was not accompanied by a corresponding decrease of respiration and glucose uptake, which were both only slightly inhibited. This interesting behavior led us to the question whether the cells loosing their viability, or whether a state is reached, which corresponds to the status of viable but non-culturable cells (VBNC, for a review see Kell et al., 1999). This question was for recombinant cells first discussed by Andersson et al. (1996b). In the frame of the actual study, analysis of the cells was extended to a much broader range of methods. These studies suggest that the overexpression of the α-glucosidase not only inhibits the growth of the host, but also leads to strong disturbances in the cell metabolism which makes the cells unable to recover. Although the molecular basis of growth inhibition has not been evaluated in detail, recent

investigations show an influence of recombinant protein production on the synthesis of cellular proteins and on cell maintenance (Dong et al., 1995, Rinas, 1996). The drastic inhibition of translation causes all cellular reactions to be inhibited depending on the stability of the most unstable protein (the limiting factor). A well-studied example of a cellular process, which is highly dependent on active translation is the initiation of chromosomal oriC replication. The initiation of oriC replication stops when the translation is inhibited by chloramphenicol addition (Clewell, 1972) or by amino acid exhaustion (Hecker et al., 1985). In this context, one might suggest that the inhibition of replication in the case of strong α-glucosidase overproduction is similarly caused by a competitive effect of the product synthesis on the synthesis of cellular proteins.

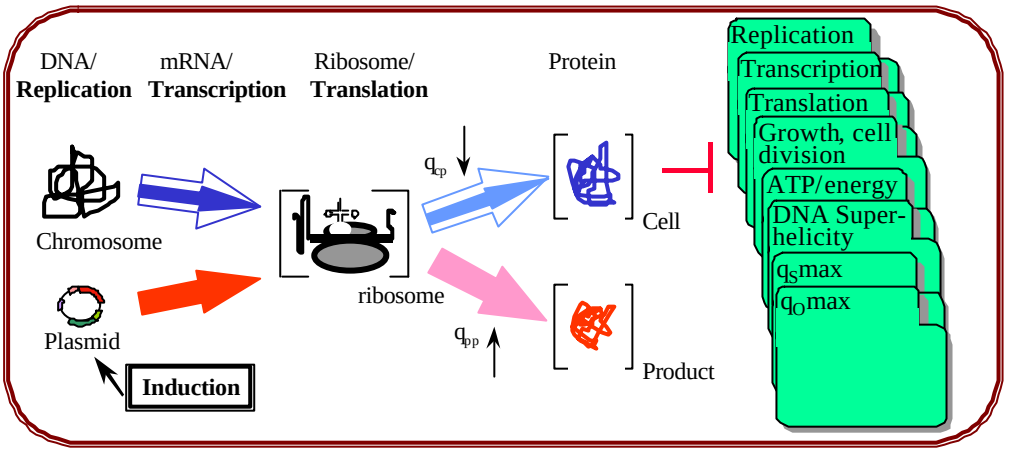


Figure 4.1: A simplified competition model of recombinant gene expression after induction. q_{cp} indicates the specific formation rate of chromosomal product for cell growth; whereas q_{pp} is the specific formation rate of recombinant plasmid product.

A simplified chart which explains this competition is shown in Fig. 4.1. In principle, a high synthesis of a recombinant protein can compete with the synthesis of cellular proteins at the level of transcription, by attracting a large number of RNA polymerase molecules to the promoter of the product gene and secondly on the level of translation, by competition for available ribosomes and translation factors. By this strong competition, the synthesis of cellular proteins decreases in an uncontrolled way, similar to a phage infection, because all cellular response to stress are based on the synthesis of a new set of proteins, and the cell has no opportunity to respond properly. Based on this assumption, the stability of different cellular metabolic reactions, such as replication, transcription, translation, glucose uptake, respiration etc., should depend on the most unstable proteinous factor. As the new synthesis of the protein is limited when translation and transcription are occupied by the recombinant product production, the most unstable factor determines the life time of the specific metabolic block. Of course, if one reaction is inhibited, other reactions will be influenced by the

network-like interconnections in the cell. As suggested from the competition model (Fig. 4.1), replication decreased within only 2 hours after induction to about 5 % of the specific synthesis rate at the induction point in both shake flask and fed-batch experiments of *E. coli* RB791 pKK177glucC with and without pUBS520. Translation was also strongly inhibited but with a slightly lower rate (50 % inhibition within 90 min). In contrast to this, transcription was rather stable in the case of *E. coli* RB791 pKK177glucC without pUBS520, where a reduction of ^{3}H-uridine incorporation to 50 % was detected within approximately 4 h after induction. However, the reduction of transcription was more stronger in the system with pUBS520.

If one considers this model, there are two major reactions which should be discussed. This is the competition for active RNA-polymerase, which surely is also effected by the actual set of sigma factors, and secondly the level of translation which is characterized by the competition of the different cellular mRNA's for competent ribosomes. Among other factors, the competition at the gene level mainly depends on the strength of promoter and the efficiency of ribosome binding side, but also on the gene codon usage, which determines the rate of translation and by this the cueing of the ribosomes on the mRNA.

From the side of vector construction, the α-glucosidase expression is controlled by a strong P_{tac} promoter and also has an efficient ribosome binding side (see Table 4.1). After induction, a major part of total protein which is produced within one hour after induction is the full length product. This evidence suggests that cellular proteins are only produced to a small part. Therefore, processes which are dependent on the new synthesis of proteins could be negatively influenced. According to our data this competition is stronger when argU-tRNA level is elevated. This behavior can be explained with the model, as the supply of this tRNA only effects the competition at the level of translation. The result of the argU supply is possibly an increase of the speed of ribosomes at the α-glucosidase mRNA. Although there is no experimental proof up till now, simulation shows that the number of attached ribosomes is much higher in this case, which would titrate most ribosomes to product synthesis and diminishes the protein flow to maintenance. As a further side effect of a higher argU-tRNA level one could speculate stabilization of the α-glucosidase mRNA by the higher coverage by ribosomes, which would potentiate the negative effect on the cellular maintenance.

Table 4.1: Basic information of plasmids pKK177glucC, pDSCrimi and encoding gene.

Plasmid	pKK177glucC	pDSCrimi
Length of DNA [base]	4675	3816
Plasmid weight [g]	4.775×10^{-18} g	3.911×10^{-18} g
Recombinant protein Product amino acids Molecular weight	α-glucosidase 591 aa 67 kDa	creatinine imino hydrolase 417 aa 45 kDa
Promoter (-35 –10 sequence)	**TTGACA**AT TAAT CATCGGCTCG **TATAAT** GTGTGGA Fusion promoter tac (trp-lac)	C T**TTGTGA**GC GGATGGC AAT **TATAAT** AGATTCA *E.coli*-phage promoter T5:P25
Ribosome binding site CCUCC (16S-rRNA)*	AATTGTGAGCGGATAACAATTTCAC ACA**GGAAA**CAGAATTATG	AATTGTGAGCGGATAACAATTTCAC ACAGAATTCATTAAAGA **GGAGA**AA TTAACCATG
First 10 amino acid codon sequence	**ATG** ACG ATA TCC GAT CAT CCA GAA ACA GAA	**ATG** CGC ATT ACA AAC GCC CAG GTT AAG AAC
Terminator	5ST1T2	T0
Origin	ColE1	ColE1
$q_{product\ (initial)}$	15 mg g^{-1} h^{-1}	40 mg g^{-1} h^{-1}
Plasmid copy number	100 –150	80 – 150

*: 16S rRNA 3'end: HO – AUUCCUCCACUAG (Neidhardt et al., 1990)

Table 4.2: Comparison of rare codons usage.

Amino acid	codon usage	number in α-glucosidase		number in CRIMI		*E. coli*	*S. cerevisiae*
Arg	AGA	15	2.54 %	2	0.49 %	0.21 %	2.09 %
Arg	AGG	4	0.68 %	1	0.24 %	0.12 %	0.95 %
Ile	AUA	6	1.02 %	1	0.24 %	0.44 %	1.84 %
Leu	CUA	4	0.68 %	4	0.98 %	0.39 %	1.35 %
Glu	GAG	12	2.03 %	10	2.20 %	1.78 %	1.95 %
Pro	CCG	2	0.34 %	3	0.73 %	2.32 %	0.54 %
Pro	CCC	4	0.68 %	11	2.69 %	0.55 %	0.69%

In contrast to the α-glucosidase system, induction of plasmid pDSCrimi with *T5*-promoter encoded protein creatinine imino hydrolase with 1 mM IPTG shows almost no cell growth inhibition. One reason might be the effect of the promoter strength. Table 4.1 shows that *T5*-promoter of pDSCrimi consists of the *–10 region* with TATAAT and the *–35 region* with TTGTGA (Shibui et al., 1988), which is weaker than the optimal consensus sequence for *E. coli* (-35 = TTGACA; -10 = TATAAT). Thereby, the transcription initiation from *T5*-promoter is lower than in the case of α-glucosidase.

Furthermore, the large-scale cultivation with a *trp*-promoter directed *E. coli* W3110 for overproducing a recombinant ZZ protein, similarly to the CRIMI process, showed that overproduction has only a slight negative effect on cell growth. There is no cell inhibition after induction with IAA, although the recombinant ZZ reached a level of 59.8 mg g^{-1} DCW.

The data obtained from mRNA analysis (Jürgen et al., submitted manuscript) show that the cellular response due to overexpression is dependent on the strength and the level of induction of the expression system. A significant transient induction of the mRNA levels of the σ^{32}-dependent heat shock genes *lon*, *dnaK* and *ibpB* after overexpression could be only observed with the highly inducible *tac*-promoter for the α-glucosidase process but not with the weaker *trp*-promoter for the ZZ process. However, the results also show that strong overexpression lead to a down-regulation of the synthesis of ribosomal proteins and proteins, which are involved in the folding of protein.

4.2 Cell segregation after induction

4.2.1 Cell segregation into viable but non-culturable cells

The growth inhibition in the α-glucosidase process is connected to a strong decrease in the ability of the cells to form colonies on agar plates. In the case of *E. coli* RB791 pKK177glucC, 3 h after induction the colony forming ability began to decrease from 100 % to about 5 % within two hours, and plasmid-free cells could be detected by replica plating from approximately 10 hours after induction. The segregation was more stronger in the strain with the plasmid pUBS520. Towards the end of fermentation, more than 70 % of the total cells carried the plasmid pKK177glucC, but have lost their ability for division in both systems.

Similar to the α-glucosidase process, cell segregation into non-culturable cells was also observed from three hours after overproduction of CRIMI. Although the rate k_d of 0.10 h^{-1} was much lower than the value in the case of the α-glucosidase process (0.35 h^{-1} for *E. coli* RB791 pKK177glucC and 0.74 h^{-1} for *E. coli* RB791 pKK177glucC pUBS), about 90 % of the total cells lost their ability for division at the end of fermentation. At this critical point where the cells began to segregate, the rate of extracellular cAMP formation was also changed. The respiration data showed also that q_{O2} and q_{CO2} began to decrease. All theses results indicated that synthesis of CRIMI effects the host cell metabolism and leads to cell segregation into non-culturable cells.

According to the experimental results, a segregated model is proposed to describe the different cell types (Fig. 4.2). This model groups the cells into four populations in dependence on their ability for cell division and plasmid maintenance. The dividing plasmid-carrying and plasmid-free cells grow with different specific growth rate (μ_{dp} and μ_d). All type of cells could lyze by different rates (e.g. k_{dp} for the dividing and plasmid-carrying cells). The non-dividing cells are arising with a certain rate (k_d) after product induction, leading first to a lower growth rate. Thus, in terms of mass fractions, the

model comprises dividing and plasmid-carrying cells ($X_{d^+p^+}$), dividing but plasmid-free cells ($X_{d^+p^-}$), non-dividing but plasmid-carrying cells ($X_{d^-p^+}$), non-dividing and plasmid-free cells ($X_{d^-p^-}$) and lyzed biomass (X_{lysis}), which originate from lysis of $X_{d^+p^+}$, $X_{d^-p^+}$, $X_{d^+p^-}$ and $X_{d^-p^-}$. Furthermore, cell segregation of the culture is designed to be induced after overexpression of recombinant gene.

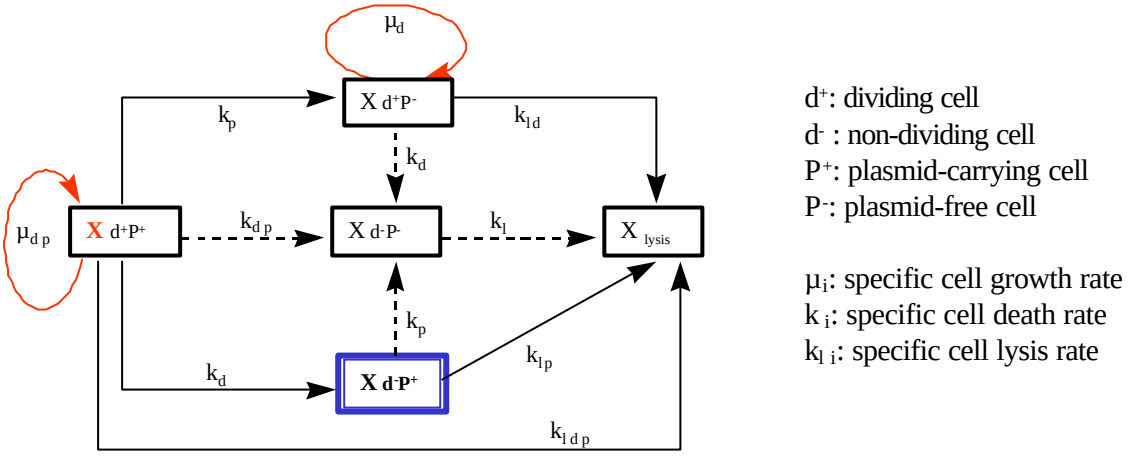


Figure 4.2: Scheme of a cell segregated model involving cell growth, cell lysis, segregation into non-dividing state and segregation into plasmid-free state.

The metabolic data suggest that the non-dividing cells ($X_{d^-p^+}$) have respiratory activity and also consume glucose. Therefore, it should be discussed, why the cells loose the ability to divide, and whether these cells are dying cells or, as discussed by Anderson et al. (1996), behave as viable but non-culturable cells. On the base of the competition model (Fig. 4.1), we propose a strong competition of the α-glucosidase product synthesis to synthesis of cellular proteins. Our analyses indicate that one of the most rapidly inhibited processes after induction of the α-glucosidase is chromosomal replication. Parallel to this a decrease in the condensation of the chromosome and the decrease of LexA is detected, which suggests DNA damage. It is well established (Walker, 1996) that the presence of lesions in the DNA is not sufficient to cause SOS induction, but the SOS signal arises when the cell attempts to replicate damaged DNA. This does not exclude, but suggests in our case no external DNA damaging agent. The SOS inducing signal is possibly created by disturbances in the DNA replication, although we do not know the limiting factor(s) yet. This is in accordance to the results published by Aris et al (1998). The authors showed by reporter gene expression experiments an induction of promoters which belong to the SOS regulon. Interestingly, they found only induction of the SOS promoters when the recombinant gene was controlled by a λP_L promoter and when the gene product was not accumulated in inclusion bodies. Indeed, in a system based on the P_{tac} promoter and mainly accumulated the product as inclusion they found no proper induction of the SOS response. However, our analytical results contradict to the conclusion of the authors that no

SOS signal occured. Rather, from the decrease of LexA we suggest that the signal is there. It might be suggested that due to the competition at the level of transcription and translation, as well as by the strong inhibition of the cellular rates of transcription and translation, a proper response is not possible and the DNA is continued to be damaged, which finally does not allow the cell to recover.

Although there are other reactions, which also contribute to growth inhibition, such as the decrease in the reaction rates of transcription and translation, the inhibition of the glucose uptake rate and the following decrease of ATP and the energy charge, all these reactions seem not to be the limiting step. They occur later than the decrease of the colony forming ability, which in the system which contains the pUBS520 plasmid starts directly after induction. Interestingly, growth inhibition and loss of the colony forming ability are much stronger in the pUBS520 plasmid-carrying strain than in the strain which does not have this plasmid. As both strains are suggested to contain the same amount of product mRNA after induction, the major competition, which finally leads to the growth arrest seems to be the competition for ribosomes. The rare AGA/AGG codons in the α-glucosidase sequence cause tailback of ribosomes and, as 10 of totally 19 rare AGA/AGG codons are contained in the first half sequence of the gene, they are suggested to negatively influence the ribosome binding rate to the ribosome binding site.

Finally, in discussion of an optimum expression system, it seems reasonable to decide not for the strongest system in consideration of construction and induction. Although large amounts of product can be obtained when the cell is dying, the uncontrolled cell death can cause pre-termination of transcription and translation, but also failure in protein folding or secretion and by this the advantage of fast expression has to be paid by higher expenses in the down-stream process.

4.2.2 Cell segregation into plasmid-free cells

Up-growth of a plasmid-free cell population is sometimes a potential problem in industrial processes with extended periods after induction of recombinant proteins. In the literature this is often considered as an effect of the recombinant protein production on the maintenance coefficient, which itself remains rather undefined (Bhattacharya & Dubey, 1995). Here we observed that rapid up-growth of a plasmid-free population is favored in the glucose limited fed-batch environment by the significantly decreased capacity for the glucose uptake in the recombinant cells, which caused an increased glucose concentration in the cultivation medium. Our analysis showed that about 30 % of the total cells were detected as plasmid pKK177glucC-free cells at the end of cultivation of *E. coli*

RB791 pKK177glucC pUBS520. In contrast to the α-glucosidase process, no up-growth of plasmid-free cell was observed in the cases of CRIMI and ZZ. The plasmid pDSCrimi and pRIT44T2 were stable after overexpression of recombinant genes.

Generally, plasmid instability is caused by the loss of the complete plasmid due to defective partitioning during cell division or alternatively due to a change in the plasmid structure by insertion, deletion, or rearrangement of DNA (Weber et al, 1989; Ryan & Parulekar, 1991; Kim et al., 1993; Lee et al., 1994; Bhattacharya and Dubey, 1996). Thereby, the following factors will influence the appearance of plasmid-free segregants: growth rate advantage of plasmid-free cells, ballast of plasmid encoded gene expression, selection pressure for plasmid-carrying cells, content of plasmid-free cells at inoculation time.

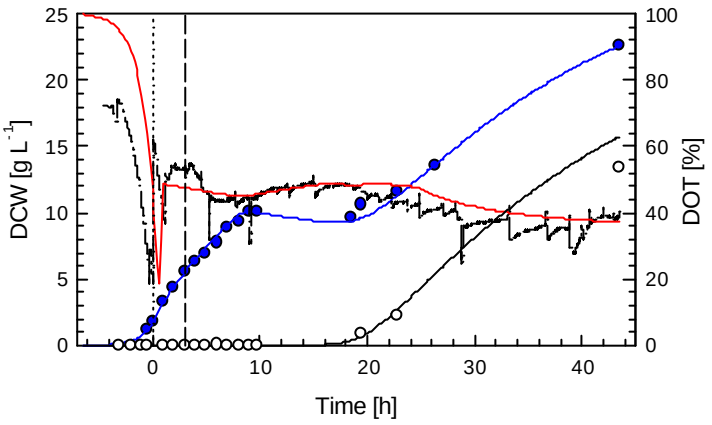


Figure 4.3: Simulation results (lines) and experimental data (● - DCW of total biomass; ○- DCW of plasmid-free cells; - · - - DOT) of fed-batch cultivation of *E. coli* RB791 pKK177glucC according to the cell segregation model in response to overexpression of recombinant gene. Zero time indicates the point of feeding start, whereas the interrupted line indicates the addition of IPTG (1 mM final concentration). Model equations and parameters used for simulation are listed in Table 4.3.*

Simulation data (Fig. 4.3) indicate that the increase in plasmid pKK177glucC-free cells is not due to plasmid loss or plasmid degradation during product accumulation. Instead, the plasmid-free cells, which segregated before induction, had the possibility to grow with the maximum growth rate when the glucose concentration increased in the cultivation medium and this sub-population succeeded to grow up to a considerable fraction of the total cells. From our experience the plamid-free cells existed already at beginning of the culture with plasmid-carrying cells together, but make up only very low proportion about 0.1 % of the total cells due to the selection pressure. The plasmid-free cells

* In Fig. 4.3, an independent inhibition of q_{Ocap} is assumed in addition to the inhibition of q_{smax}.

can only overgrow if the growth of plasmid-carrying cells and glucose uptake capacity was inhibited after induction.

Table 4.3: Segregation model and parameters of the aerobic growth of *E. coli* on glucose which has been used to describe cell segregation to plasmid-free state and lysis after induction during fed-batch cultivations.†

S_0 [g L^{-1}] = 5.3;	K_La = 400
DOT_0 [%] =100;	H = 14000
V_0 [L] = 4;	DOT_{st} [%] =100
F [L h^{-1}] = 0.053;	V_{sample} [L h^{-1}] = 0.02
S_i [g L^{-1}] = 200	
$q_s = q_{smax} \times S / (S+K_s)$;	$q_{sx} = q_s - q_m$
$\mu = q_{sx} \times Y_{xsem}$;	$q_o = q_{sx} \times Y_{osgrowth} + q_m \times Y_{osmaint}$

$dV/dt = F - V_{sample}$

$dS/dt = F/V \times S_i - F/V \times S - q_{sp1} \times X_1 - q_{sp2} \times X_2$

$dDOT/dt = K_La \times (DOT_{st}-DOT) - q_{op1} \times X_1 - q_{op2} \times X_2 \times H$

	P1 (X1)	P2 (X2)
X_0 [g L^{-1}]	0.01	0
K_s [g L^{-1}]	0.035	0.035
q_m [h^{-1}]	0.05	0.05
Y_{xsem}	0.46	0.5
q_{smax} [h^{-1}]	1.5	1.5
$Y_{osgrowth}$	1.07	1.07
$Y_{osmaint}$	0.1	0.1
q_{I1} for μ	0.00025	
q_{I2} for q_s	0.0002	
q_{I3} for q_s	0.00055	
r_{Seg} [%]	0.0008	
q_{smax} after ind. =	$q_{smax} / (1+q_{I2} \times (Time-Itime^a)/ q_{I3})$	
μ after ind. =	$q_s \times Y_{xsem} - q_{I1} \times \exp(Time-Itime)$	
dX_{P1}/dt =	$-F/V \times X_{P1} + \mu \times X_{P1} - r_{Seg} \times \mu \times X_{P1}$	
dX_{P2}/dt =	$-F/V \times X_{P2} + \mu \times X_{P2} + r_{Seg} \times \mu \times X_{P1}$	

[a]: Induction time point, in this case Itime = 3 h

Furthermore, the simulations show that the experimental data for the loss of the colony forming ability and the up-growth of the culture by plasmid-free cells can be fitted only by correction for the distinct q_{scap} value. This is interesting and was not expected, because overexpression of α-glucosidase influences a number of different processes, such as transcription, translation, replication, represses cellular stress responses etc. However, the effect on the glucose uptake is quite crucial for the cell and the fermentation process. As is evident from Figs. 3.17-18, the decrease of q_s is directly connected to the decrease in the respiration rate and correspondingly to a lower ATP production.

† In Table 4.3, the equations are only shown for one sub-population, but were defined in the model for two populations, namely plasmid-carrying and plasmid-free cells. However, both populations are considered in the differential equations. In this case, the parameters carry the subscripts P1 for plasmid-carrying cells, and P2 for

4.3 Plasmid content after induction

The number of gene copies in a cell influences gene expression. The results show that ColE1 related plasmids are amplified during the production of recombinant proteins as long as product synthesis leads to growth inhibition. Plasmid amplification was observed for different products and was found to be independent of the strain and the inductor, but depends on the replication mode of the plasmid as we discussed extensively in a recent paper (Teich et al., 1998). Plasmid amplification seems to be directly connected to the inhibition of cellular growth, since no plasmid amplification was observed in systems for which the formation of high levels of recombinant product did not affect growth, such as overproduction of CRIMI, ZZ protein, GroES and GroEL. However, a slowly declining growth rate alone, for example by constant feed supply in a fed-batch cultivation, does not yield a high plasmid concentration, because normally cell synthesis processes are exactly tuned. In contrast, after induction of a recombinant protein, when the chromosomal replication is inhibited by the factors discussed above, plasmid replication proceeds for a certain time.

This is also in agreement with the proposed competition model. Namely, it is known that by methods, which lead to a down-regulation of translation and further more suppress the natural response of the cell to this event, which is the formation of the alarmone ppGpp, lead to plasmid amplification. This is because plasmid amplification is much less dependent on proceeding translation than the initiation of chromosomal DNA replication. This has been extensively discussed in relation to the effect of chloramphenicol and the use of *E. coli* relA mutants as hosts for efficient plasmid production (Hecker et al., 1985).

In the recent paper (Teich et al., 1998), we discussed this plasmid amplification by recombinant protein induction in connection to eventually beneficial effect on product formation. I want to highlight again that this amplification seems to be a side effect connected to the decrease of many cellular activities. Especially, as plasmid amplification occurs as a result of the down-regulation of translational activity, it should be hardly possible to take advantage of the high gene copy number.

4.4 Stress responses during fed-batch cultures of recombinant E. coli

An appropriate response is essential for effective adaptation of bacteria to changes in their natural environment. This response is mediated by global regulatory mechanisms affecting several pathways that differentially turn many genes on or off in response to environmental stimuli. All the responses

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cells which have lost the pKK177glucC plasmid. Parameters for population P1 were calculated from batch data,

which are stimulated by one environmental signal are defined as a “stimulon”, whereas the term “regulon” is used for a cascade which is regulated by one special cellular response regulator (Fig. 4.4, also see Hecker et al., 1988; Neidhardt et al., 1990). For example, the stringent response by ppGpp, the general stress response by σ^S and the cAMP/CRP response are three regulons which are induced by the stimulus glucose starvation (Gottsman, 1984; Matin, 1989,1991,1992; McCann et al., 1991; Hecker et al., 1996; Saier, 1996; Hengge-Aronis, 1999). By this logistics it has to be taken into consideration that many genes are regulated by different factors and therefore the whole has to be seen as a network (Fig. 4.5, also see Matin, 1991, 1992; Cashel, 1996; Hengge-Aronis, 1996).

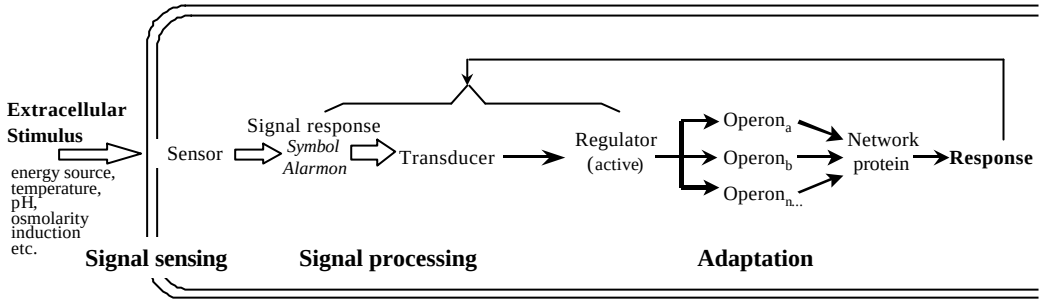

Figure 4.4: Gene network depicted as a stimulus-response pathway.

4.4.1 Stress responses to glucose limitation/starvation

As mentioned above, the entry of *E. coli* into glucose limitation/starvation is connected to the induction of the stringent response, the general stress response and the cAMP-CRP mediated response, which together induce more than 100 proteins in a temporally regulated mode (Fig. 4.5; also see Neidhardt et al., 1990, McCann et al., 1991; Schultz & Matin, 1991; Hengge-Aronis, 1999). As described in Fig. 4.5, The cAMP/CRP mediated response could be understand as an offensive strategy by which metabolic genes are induced which give *E. coli* the possibility to open up new nutrient sources, but it also stimulates the expression of proteins involved in motility and chemotaxis (Saier, 1996). The stringent response as well as the general stress response are both defensive strategies and known to be necessary for cellular adaptation mechanisms to stress and starvation, which are not only activated due to starvation, but also to other signals like changes in the growth rate (Zgurskaya et al., 1997), heat-shock (Cashel et al., 1996; Muffler et al., 1997), and hyperosmotic shock (Lange & Hengge-Aronis, 1994; Hengge-Aronis, 1996b). Furthermore, the

whereas the parameters for population P2 were assumed.

general stress response is important for the cross-protection of the cell to other stresses (Jenkins et al., 1988).

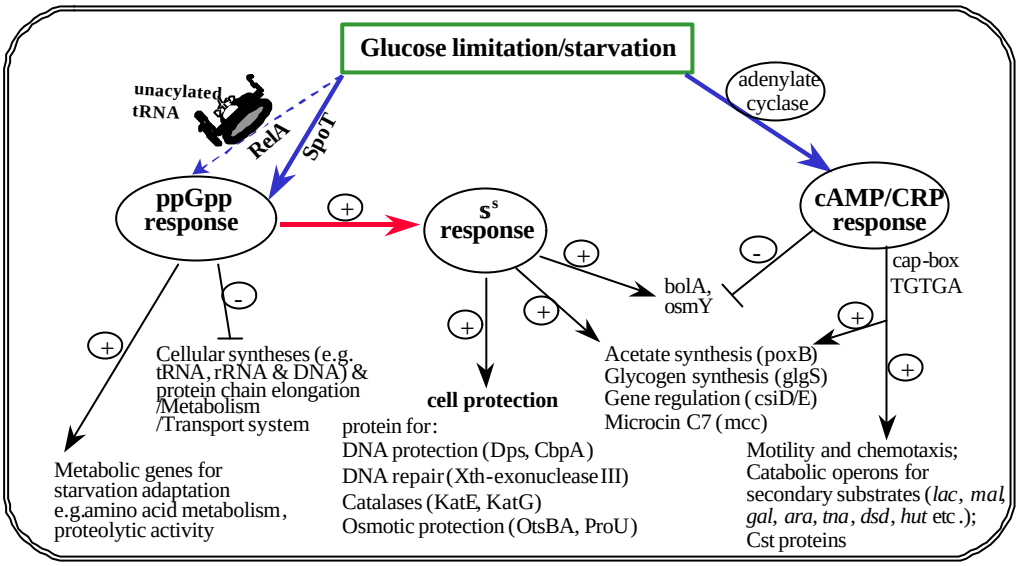

Figure 4.5: Three stress responses to glucose limitation and starvation in *E. coli*. Positive and negative influences are indicated by arrows and bars respectively.

During the glucose limited fed-batch cultivation of *E. coli*, PpGpp accumulated transiently after the nutrient shift from the batch phase to the glucose limited fed-batch phase. Afterwards the level of ppGpp was approximately constant at a level, which was two to three times higher than during unlimited growth (see Fig. 3.23a).

Similar to ppGpp, σ^S increases when glucose becomes limiting. However, the response is slower and σ^S only increases 4- to 5-fold. The level during the fed-batch phase is about 2.5-fold higher than during the batch (see Fig. 3.25a). ppGpp and σ^S respond to abrupt changes in the glucose concentration with different kinetics. The transient increase of ppGpp is much shorter and more significant at the onset from exponential growth to limited growth in fed-batch cultivation. A lower response at a lower pre-shift growth rate has been observed in stopped-flow experiments (Fig. 3.24). Furthermore, the investigation of both response regulators ppGpp and σ^S during glucose-limited growth at different growth rates and with different cultivation strategies support the assumption that the stringent response regulator ppGpp has an effect on the general stress response σ^S (Fig. 4.5, also see Gentry et al., 1993). However, during transients with slow continuous changes of the nutrient availability, this concerted reaction of ppGpp and σ^S is less apparent, indicating the specific importance of these regulators for the adaptation of the cells to fast changes of environmental parameters (for further discussion see Teich et al., 1999).

cAMP is a master regulator which accumulates intracellularly following glucose starvation, but is also released in the cultivation medium. The extracellular concentration of cAMP also increases after glucose limitation and often further accumulates up to the end of the fermentation (see Fig. 3.30a).

In connection to the industrial application of nutrient-limited growth strategies, our results indicate that changes in the availability of nutrients cause cellular responses in the time windows of minutes. Although the response of ppGpp occurs first, σ^S protein formation is only delayed for a few minutes. On the other hand, as we discussed in a recent paper (Teich et al., 1999) slow continuous changes cause adaptation which is different from what is usually called a stress response. This has to be considered if, for example, stress promoters are used for the expression of recombinant gene products which are thought to be automatically induced at a certain growth rate. For instance, the *phoA* promoter which is induced by phosphate starvation has often been used for production of secreted proteins (e.g. active F_{ab} fragment in the periplasm of *E. coli*, Carter et al., 1992; Georgiou, 1996). Another interesting expression system working at low growth rates has been described by Schroeck et al. (1992). The authors cloned a *cap* region, which is responsible for the binding of the catabolite activator protein (CRP). This unit relates the expression of the target gene to cAMP concentration and considerably increases the production of interferon $\alpha 1$ with decreasing dilution rate for a long time interval (20 h) when the expression is *trpP.O* controlled.

Moreover, the mRNA data analysis shown in Fig. 3.29a-e adds information on the behavior of a set of genes belonging to the general stress response. The level of *rpoS* mRNA increases shortly after the transition from the unlimited batch phase to the glucose-limited fed-batch phase, which corresponds to the data obtained by the immunoblot for σ^S and by the analysis of the positive regulator ppGpp (Figs. 3.25a and 3.23d). In contrast to the *rpoS* mRNA, the level of the *osmY* mRNA only increases after a delay of eight hours, possibly because it is regulated by additional factors, such as cAMP. As a control the measure of a further gene, the *rpoA* mRNA, coding for the α-subunit of the RNA polymerase was included, because it is assumed that ist transcription is not controlled by additional factors. Although a rather constant level of *rpoA* mRNA was found throughout the growth limited fed-batch period (Fig. 3.29e), the level of this mRNA was increased approximately fivefold during the preceding batch phase (data not shown), where the cells grew at the maximum specific growth rate. As the mRNA data were normalized to the total amount of RNA, one can assume that this drop of the *rpoA* mRNA level during the transition from unlimited growth to glucose limitation is correlated to the change of the RNA pool and the activity of transcription.

In summary, our results demonstrate that the recombinant *E. coli* cells in a fed-batch cultivation respond to the stress of glucose limitation by inducing the stringent response, the general stress response and the cAMP/CRP mediated response. The response of the three regulators was clearly shown in situations where a nutrient shift occurs.

4.4.2 Stress responses after the induction of recombinant genes

After induction of α-glucosidase, we found a decrease of ppGpp below the detection limit (Fig. 3.23c-d). Similarly, also the level of σ^S decreases significantly. The mRNA analysis showed that this decrease is due to lower synthesis of σ^S-mRNA and not due to a lower σ^S stability. Following α-glucosidase induction, no induce of σ^S-dependent genes was detected. We suggest that the failure to perform the σ^S response after induction of the recombinant genes might be suggested to contribute to the high rate of cell death. In accordance with the σ^S data, the expression of the σ^S dependent gene *dps* (*pexB*) seems to be down-regulated under overproducing conditions. The 2D PAGE analyses revealed a weak but reproducible decrease in the Dps protein level in the overproducing strain (Jürgen et al., submitted manuscript). Similarly, we also found that *osmY* mRNA is not induced after induction of α-glucosidase.

The data obtained from a *clpP rpoS* mutant indicate that the general stress response regulated by the alternative sigma factor σ^S plays a role in the survival during the production of recombinant proteins in *E. coli*, in which a similar α-glucosidase mRNA level was found two hours after induction as in the wild type. Although the wildtype strain had a similar α-glucosidase mRNA level as the *clpP rpoS* mutant, the mutant produced much less α-glucosidase than the wildtype (Fig. 3.27). Interestingly, the death rate of this mutant (k_d = 0.79 h^{-1}) was almost the same as of wildtype (k_d = 0.74 h^{-1}) within the first 6 h after induction and stayed at this level to the end of cultivation, whereas the death rate of wildtype decreased to a value of 0.25 h^{-1} at the time point of 6 h after induction. Concomitantly a *clpP* mutant, which has a higher level of σ^S clearly shows a higher viability, although in this case it is not clear whether this effect is due to the function of the σ^S response, or due to the lower production of α-glucosidase. However, since the production of the α-glucosidase was not improved in the *clpP rpoS* double mutant, but strongly decreased, we conclude that a certain level of σ^S response before induction is favorable, in order to maintain the house-keeping functions of the cell – measured as viability – over a longer time.

Table 4.4: Stress factors after induction of α-glucosidase during fed-batch fermentation.

	Protein level after induction	mRNA level after induction	Function and response system
ppGpp	decreased below the detection limit	-	stringent response regulon
cAMP	decreased	-	cAMP/CRP mediated response regulon
rpoS (σ^S)	decreased	decreased	general stress response regulon
dps (*pexB*)	weakly decreased	n.d.	σ^S dependent gene coding for catalase
osmY	n.d.	decreased	σ^S dependent gene
lon	n.d.	a significant but transient increase	σ^{32} dependent heat shock gene (protease)
dnaK	2-3 fold increased	a significant but transient increase	σ^{32} dependent heat shock gene (chaperone)
GroEL	2-3 fold increased	increased	σ^{32} dependent heat shock gene (chaperone)
IbpB	high amount in inclusion bodies	the strongest increase and remained at a clearly higher level	heat shock gene coding for the inclusion body associated protein
rpoA	n.d.	drops to 50 % within 1 h a.i.	σ^{70} dependent gene coding for the α-subunit of the RNA polymerase
tig & *ppiB*	n.d.	decreased	σ^{70} dependent genes coding for peptidyl-cis-trans-isomerases
htrA	n.d.	decreased	σ^{24} dependent gene
lexA	decreased	n.d.	SOS-response protein

n.d.: not determined; a.i.: after induction.

Furthermore, our data show that a high level of σ^S is not favorable for genes which are regulated by σ^{70}-RNA polymerase, as σ^S competes to σ^{70} at the level of transcription. On the contrary, the decrease of the σ^S level, either in a *rpoS* mutant or by avoiding synthesis of ppGpp, the positive effector of σ^S synthesis, favors the formation of σ^{70}-RNA polymerase holoenzyme. This increases the transcription of σ^{70} regulated expression systems like the P_{tac} system, which consequently may result in a higher expression of the recombinant gene. This is in accordance with the study of Chou et al. (1996) who obtained higher product formation in a *rpoS* mutant. The data are also consistent with the results of Dedhia et al. (1997) who found a threefold increase of CAT mRNA levels in a ppGpp deficient strain, as the level of σ^S should be lower in this strain. It is supposed that a major bottleneck of recombinant protein production is the competition of the heterologous mRNA with the homologous mRNAs at the level of translation (Dong et al., 1995; Rinas 1996). However, our data indicate that the competition of different sigma factors for RNA polymerase has also to be considered in the production of recombinant proteins. The concentration of the different sigma factors seems to be of regulatory importance for the gene expression pattern in general (Farewell et

al., 1998; Jishage et al., 1995, 1996) and should be considered for the choice of an expression system and in process design. Beside the down-regulation of σ^S during overexpression, we also found a significant reduction of the *rpoA* mRNA level shortly after induction of the *tac*-promoter based expression system, which appears to be not related to the σ^S response.

The data listed in Table 4.4 also demonstrate that *E. coli* responds to a strong overexpression by a heat shock like response. The mRNA levels of heat shock genes like *dnaK* and *lon* were significantly increased. It is supposed that the cellular concentration of the heat shock sigma factor σ^{32} is influenced by the intracellular level of heterologous proteins (Grossmann et al., 1985; Enfors, 1992; Kosinski et al., 1992b; Sherman & Goldberg, 1992; Harcum & Bentley, 1999), but the higher transcription could also be a result of activation of σ^{32}. It has been shown that heat shock in *E. coli* causes a transient stabilization of the heat shock sigma factor σ^{32} and thus a temporary increased transcription of heat shock genes (Nagai et al., 1991), which might be the case in our system. However, despite the observed transient increase of the mRNAs of the heat shock genes *dnaK* and *lon*, the 2D-PAGE analyses of this and other studies demonstrate that this transient response is enough, to elevated the level of the heat shock proteins significantly for a longer time.

Furthermore, besides these responses, after α-glucosidase induction, the level of extracellular cAMP began to decrease about 2 hours after induction of α-glucosidase (Fig. 3.30c), where we are not yet sure, whether this effect influences the cells or this is only a side reaction.

Besides the behavior of the cells in connection to the stress responses, in evaluation of AXP level and the respiration data from the frequent sampling experiments presented in Fig. 3.17-18, we suggest that the early production of α-glucosidase causes a redirection of the metabolic carbon flows. As the feeding rate is constant, the increase of q_O and q_{CO2} at one hour after induction must be related to a redirection of the carbon flow, possibly a higher throughput of the glucose through the glycolysis. However, after the transient increase, we observed that the specific oxygen uptake capacity declines. This effect was described earlier by Zabriskie et al. (1987) and is possibly related to the negative effects of strong synthesis of a foreign protein on the cellular maintenance.

The ATP energy level was significantly influenced by the induction of α-glucosidase, where we found a quick increase of the ATP level for a short period after induction. Then the ATP level sharply decreased which was connected to a significant increase of the ADP and AMP levels due to the RNA degradation (Fig. 3.17). By this behavior, the energy charge and the ration of ATP/ADP was drastically reduced, which has to be seen as an important factor in the inactivation of the cellular

reactions. Furthermore, the reduce of the ATP level was connected to a decrease of the specific oxygen uptake rate, which finally was caused by a strong inhibition of the glucose uptake at this time.

Interestingly, in difference to the α-glucosidase system, the general stress response was strongly induced after overexpression of recombinant CRIMI (Fig. 3.28), where also no cell growth inhibition occurred. The difference between both systems was also observed in connection to the accumulation of cAMP (Fig. 3.30c and 3.31). Furthermore, the recent literature shows that recombinant protein production also induced the SOS response (Aris et al., 1998) and the stringent response (Cserjan-Puschmann et al., 1999) for a transient period. Indirectly, the studies with mutants of the general response regulators of the stringent response and the general stress response by Dedhia et al. (1997) and by Chou et al. (1996) suggest that these responses are somehow connected to the induction of recombinant proteins.

In summary, the σ^S stress response seems to be of secondary importance for the survival of *E. coli* during strong overexpression of recombinant genes. These results indicate that the competition of different sigma factors for the efficient initiation of transcription is of importance for the overexpression of recombinant proteins These results also demonstrate that the analysis of mRNAs is a potential tool for monitoring of bioprocesses to detect bottlenecks in a process which should thus lead to further process optimization.

4.5 Cell physiology in large-scale bioprocesses

The data obtained from the large-scale glucose limited fed-batch cultivations of *E. coli* W3110 with and without pRIT44T2 demonstrated the existence of substrate gradient, oxygen and pH-gradient. The comparison of cultivation ET01 with a 10 L lab-scale cultivation by Xu and coworkers (1999) showed following typical differences: (1) substrate gradients in time and space were presented which ranged from 20 mg L^{-1} to 200 mg L^{-1}; (2) over-flow metabolic by-products such as formate are observed during a certain phase of the fermentation, which were not observed at a lab scale; (3) the overall biomass yield was reduced by 24 % in large-scale process (Xu et al., 1999). All these phenomena belong to the “scale-up effect” caused by limitation of power input, transport and mixing. Therefore, the individual cell is exposed to an oscillating micro-environment while circulating in the large-scale reactor. At the small scales of laboratory and pilot plant reactors good mixing and high mass transfer can be easily achieved by increasing the specific power input (P/V) to the reactor. In order to study the scale-up effect in bioprocesses on a quantitative level, a model of fluid mixing and

microbial kinetics was developed by the cooperation partners of the EU project "Bioprocess scale-up strategy" (see project reports and Vrábel et al., 1999a,b).

To reduce the gradients and the causing effects, strategy from microbial aspect and mixing side can be taken. On the mixing side, the introducing of Scaba impeller can improve the overall biomass yield to 0.36 from 0.28 (Table 3.2). The analysis of metabolic by-products such as acetate, lactate and formate by Xu et al. Within this EU project showed that the repeated production and re-assimilation of these compounds may be responsible for the reduction of biomass yield. Furthermore, oxygen gradients were also formed due to insufficient mass transfer and/or from increased respiration in zones with locally high glucose concentration. Interestingly, total cell number and cfu analysis showed no cell segregation during all three large-scale processes of *E. coli* W3110 in 30 m^3 fermenter, which suggests that fluctuating metabolic rates under strong limitation may prevent cell segregation. A higher cell lysis was observed in exponential growth phase where the extracellular protein made up to 5 % of the total protein.

However, a significant difference in cell viability was detected between the both fermentations for overproduction of recombinant ZZ protein (Fig. 3.39d). The cell segregation was affected by stirrer configuration. A different mixing efficiency and flow pattern existed between Rushton stirrer set and Scaba stirrer set. In fermentation PU01 with Rushton stirrer set, the number of cfu decreased to about 30 % of the total cells in the end of the experiment after induction. Cell segregation into viable but non-culturable cells has reported by Andresson et al. (1996b) in high cell density cultures performed at low specific growth rate both in induced and uninduced systems. Flow cytometric analysis of cells in broth samples, based on two multi-staining protocols, revealed a progressive change in cell physiological state throughout the course of the fermentations (Hewitt and Nierow, 1999). From these measurements they concluded that the loss in reproductive viability towards the end of the fed-batch process is due to cell death and not due to the formation of a VBNC as had previously been reported. In contrast to this no cell segregation was found in the fermentation PU02 with the Scaba stirrer set.

4.6 Influence of substrate oscillations

The influence of substrate oscillations are known to occur in large-scale cultures (Bylund et al., 1998, Larsson et al., 1996, Xu et al., 1999) on recombinant fed-batch processes of *E. coli*. Although this study was performed in a model reactor situation by an oscillatory feed protocol and would have to

be extended in the future to analyses in large-scale processes, this study provides useful information on which kind of responses should be looked at in the large scale.

Oscillatory cultures are usually neither well understood nor desirable. In contrast, the synchronized cultures permit study of cell cycle dependent events and relations under well controlled and reproducible cultivation conditions (Sonnleitner, 1996). The most interesting result was that the up-growth of a culture by a plasmid-free population can be influenced by the time interval during which the cells are exposed to glucose starvation. The slower up-growth of a plasmid-free population under fermentation conditions, where oscillations occur or where zones and gradients exist, could be seen as a positive influence of oscillations. Furthermore, oscillations contribute to a pre-adaptation of the cells to a stress situation, which could induce a co-adaptation to other stresses occurring in the bioreactor environment. This could have a positive influence on the viability of the cells by induction of general stress responses, such as the stringent response and the general stress response and would possibly adapt the cells not only to starvation, but also increase the tolerance level to osmotic stress, pH stress, and high temperatures. However, on the other side, oscillations were also seen to affect product yields in a negative fashion. This is possibly caused by a higher level of proteolytic factors, which especially affect products which are formed as soluble proteins, but could also contribute to degradation of proteins in inclusion bodies.

Furthermore, the increased formation of carbon dioxide from glucose under oscillating conditions can be a drawback in certain conditions, as it decreases biomass formation due to a lower flow of glucose to cellular syntheses. Interestingly, our results showed oxygen consumption was not influenced by oscillations, which could indicate that energy limitations did not occur. Although the increased formation of carbon dioxide was a positive aspect under the conditions of the present study, the lower maximum growth rate of the plasmid-free population indicates that, under conditions where growth is important, nutrient oscillations should be avoided. In discussing the effect of oscillations on cells circulating in a large-scale bioreactor it should be noted that the data discussed here are specific to defined oscillations. The situation in a large-scale bioreactor is much more complex since many parameters may be oscillating. As a result, knowledge-based process optimization, including extensive biological data, will be possible based on such studies.

5 Conclusion

From the investigation of cellular responses to the induction of recombinant genes in fed-batch culture of *Escherichia coli*, by analyzing a number of parameters such as cell growth, plasmid stability and amplification, recombinant protein formation, cellular metabolism, and several stress responses, it is obvious that induction of recombinant genes causes a competition with house-keeping cell functions at the transcriptional or the translational level, for the RNA polymerase molecules, available ribosomes and translation factors. By this strong competition, a decrease of different cellular metabolic reactions, such as replication, transcription, translation, glucose uptake and respiration was determined directly after induction of our model system α-glucosidase. I assume that this competitive effect leads to the inhibition of cell growth. Thereby, the host-cells segregate into viable but non-culturable cells (VBNC) which show metabolic activity but lost their ability to recover. The ability of these cells for replication is apparently not only impaired by competition of the synthesis of the recombinant product to the formation of cellular house-keeping proteins, but specifically by continued damage of the chromosomal DNA which results in loss of DNA density.

Furthermore, the cell growth and the behavior of general stress regulators of *E. coli* was compared in two different recombinant processes which are both characterized by the accumulation of the product in inclusion bodies to similar amounts. From the levels of the response regulators in connection to the data from mRNA analysis and 2-D-gel electrophoresis, we conclude that after induction of the α-glucosidase, the general stress response is not induced. In difference, a corresponding response is induced in the CRIMI process. Suggestively, the different behavior of the two processes in connection to the survival of the cells and the maintenance of the metabolic activities (glucose uptake and respiration, but also activity of replication, transcription and translation) are a consequence of the different behavior in correlation to the induction of the general stress response. Moreover, the different behavior of processes in relation to these responses may influence the length of the production phase, the up-growth of plasmid-free cells, and even effect the product quality. The fast inhibition of all cellular reactions in the α-glucosidase process might be responsible for the poor quality of the α-glucosidase-IB's, which contain a high amount of product fragments.

Finally, evaluation of the large-scale experiments (12 m^3 / 30 m^3) whit a broad spectrum of analytical methods demonstrated the existence of gradients for glucose and oxygen and the influence of mixing efficiency on cell growth and product formation. Therefore, it is necessary to integrate the microbial physiology and fluid dynamics, in order to develop new scale-up methodologies and tools for microbial bioprocesses.

6 ACKNOWLEDGEMENTS

This work has been performed at the Department of Biochemistry and Biotechnology of the Martin-Luther-Universität Halle-Wittenberg in Germany. I am sincerely grateful, that it was given me an opportunity to be a part of the atmosphere of knowledge, collaboration, encouragement and friendship.

First of all, I want to express my enormous gratitude to my supervisors, Prof. Rainer Rudolph and Dr. Peter Neubauer, for permitting me to do this research work, for providing such a wonderful research environment, and for guiding me with their deep knowledge in modern biotechnology. Especially, to Dr. Peter Neubauer, for helpful suggestion and discussion, for having the time to read and revise this thesis step by step, for the experimental analysis to determine the replication, transcription, and translation rates.

Dr. Sylke Meyer, for some ppGpp analysis; Antje Teich, for some plasmid- and σ^S-quantification of *E. coli* RB791 pKK177glucC; Stephan Riemschneider, for the S8-protein analysis; and all members of the fermentation group, for the cooperation and useful discussion.

Uta Best, for the excellent technical assistance; Silke Nicklisch, for the LexA-protein analysis.

Jeannette Winter, Antje Breitenstein and Dr. Vlada Khalameyzer, for their critical reading of this thesis.

Dr. Elisabeth Schwarz, Dr. Gerald Böhn and Dr. Hauke Lilie, for their advice and a wide scope of knowledge.

Ulla Grauschopf, Constanze Franz, Sabine Meyenburg, Uli Schmidt, and all of our institute for helping me to work in the area of molecular biology etc. ...

Prof. Michael Hecker, Dr. Thomas Schweder, Britta Jürgen from the Institute of Microbiology at the University of Greifswald, for the continuous cooperation, the mutant strains, and the analysis of mRNA and 2D-eletrophoresis. Dr. R. Hanschke, for the analysis of cell samples with transmission electron microscopy.

Also, I would like to thank the people, who worked for the same EC project and performed those large-scale experiments. They are: Prof. S.-O. Enfors, Prof. L. Fuchs, Prof. G. Hamer, Prof. M. Hecker, Prof. B. Hjertager, Dr. S. Hjorth, Ir. F. W. J. M. M. Hoek, Prof. K. Luyben, Dr. Å. Manelius, Prof. A. Nienow, Prof. M. Reuss, Dr. H. Skogman, Prof. C. Trägardh, Dr. R. von der

Lans, Dr. T. Schweder, G. Blomsten, L. Boon, Dr. P. C. Friberg, Dr. C. Hewitt, S. Hjoort, M. Jahic, B. Jürgen, T. Kovacs, Dr. E. Krüger, Dr. N. Noisommit-Rizzi, Dr. D. O'Beirne, A. Rozkov, F. Studer, F. van der Schot, Dr. P. Vrabel, and Dr. B. Xu. The group of G. Larsson (KTH, Stockholm), for performing the fed-batch cultivations of MC4100 $relA^+$.

Furthermore, E. Kopetzki, U. Brinkmann, R. Müller and Roche Diagnostics (Werk Penzberg, Germany) for the plasmids (pKK177glucC, pUBS520, pDSCrimi), the *E. coli* Stock Center (New Haven, USA) for the strains *E. coli* RB791 and W3110. Especially, I thank Prof. R. Hengge-Aronis (Humboldt Univ. Berlin, Germany) for the generous gift of σ^S antibodies, Prof. Iohihama (Mishima, Japan) for the σ^{70} antibodies, Prof. E. Bremer (Univ. Marburg, Germany) for H-NS antibodies and control strains MC4100 (hns^+) and PD32 (hns^-), Prof. J. W. Roberts (Univ. Cornell, Ithaca, U.S.A.) for LexA antibodies and Dr. Brimacombe (Univ. Berlin, Germany) for S8 polyclonal antibodies and the ribosome standard.

This work was financially supported by the Biotechnology program of the European Community (Projects No: BIO4-CT95-0028, BIO4-CT98-0167), a grant from Sachsen-Anhalt for the period from April 1996 to March 1998 and a grant from the Hanns-Seidel-Foundation for the period from September 1995 to February 1996.

Finally, very special thanks to my husband Tao Liu and my daughter Su Liu for their encouragement, support and love.

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II. Teich, A., Meyer, S., Lin, H.Y., Andersson, L., Enfors, S.-O. and Neubauer, P. (1999) Growth rate related concentration changes of the general stress response regulators σ^s and ppGpp in glucose limited fed-batch and continuous cultures of *Escherichia coli*. Biotechnology Progress, *15*, p123-129

III. Lin, H.Y. and Neubauer, P. (2000) Influence of controlled glucose oscillations on a recombinant fed-batch process of *Escherichia coli*. J. Biotechnol. in press

IV. Schweder, T., Lin, H.Y., Jürgen, B. Riemschneider, S., Khalameyzer, V., Gupta, A. and Neubauer, P. (2000) Recombinant overexpression in *clpP*-deficient *Escherichia coli* strains: effects on growth and product synthesis. submitted manuscript

V. Jürgen, B., Lin, H.Y., Riemschneider, S., Scharf, C., Neubauer, P., Hecker, M. and Schweder T. (2000) Monitoring of genes that respond to overexpression of recombinant protein in *Escherichia coli* fed-batch process. submitted manuscript

VI. Lin, H.Y., Hanschke, R., Nietsche, T., Jarchow, R., Schwahn, C., Nicklisch, S., Hecker, M. and Neubauer P. (2000) Strong overexpression of recombinant genes in *E.coli* triggers DNA relaxation and cell death. submitted manuscript

VII. Lin, H.Y., Mathiszik, B., Xu, B., Enfors, S.-O. and Neubauer, P. (2000) Determination of the maximum specific uptake capacities for glucose and oxygen in glucose limited fed-batch fermentations of *Escherichia coli*. submitted manuscript

VIII. Lin, H.Y. and Neubauer, P. (2000) Kinetics of the general stress response regulators ppGpp, σ^S and cAMP after induction of recombinant genes in glucose limited fed-batch cultivations of *Escherichia coli*. manuscript

IX. Lin, H.Y., Blomsten, G., Riemschneider, S., Meyer, S., Jürgen, B., Xu, B., Noisommit-Rizzi, N., Rozkov, A., Schweder, T., Enfors, S.-O. and Neubauer P. (2000) A kinetic study of cell growth in large-scale fed-batch bioprocesses of *Escherichia coli*. manuscript

X. Lin, H.Y., Mathiszik, B. and Neubauer, P. (2000) Inhibition of the cellular capacity for glucose uptake and respiration in response to induction of recombinant gene expression favors up-growth of plasmid free segregants. manuscript

Erklärung

Ich erkläre hiermit, daß ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfaßt, andere als die von mir angegebenen Quellen und Hilfsmittel nicht verwendet und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als kenntlich gemacht habe.

Die Arbeit wurde bisher an keiner anderen Hochschule oder Universität zur Promotion eingereicht.

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