Processes for the Production of Pharmaceutical Grade Plasmid DNA

Habilitation Thesis

(Habilitationsschrift)

submitted by

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Bielefeld

June, 2008

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It was the best of times, it was the worst of times,...

It is a far, far better thing that I do, than I have ever done; it is a far, far better rest that I go to, than I have ever known.

Charles Dickens, A Tale of Two Cities

Acknowledgements

My deepest gratitude goes to Prof. Dr. Erwin Flaschel. During my time at the Chair of Fermentation Engineering at Bielefeld University, he introduced me to biotechnological research and I was able to participate from his vast knowledge in this field. I also thank him for his continuing support of my work and for his efforts put in a successful development of my professional career. In addition, his understanding for non scientific issues is also gratefully acknowledged.

Secondly, I would like to extend my deepest gratitude to Prof. Milton T.W. Hearn of the ARC Special Research Centre for Green Chemistry at Monash University, Clayton, Australia for further developing my career on the international level. His hospitality and support during two research visits to Monash University is also gratefully acknowledged. His scientific career as well as the interdisciplinarity of the research centre under his supervision serve as a role model for my professional development. I also wish to thank him for examining this thesis.

Furthermore, I would like to thank Prof. Dr. Thomas Noll, Chair of Animal Cell Culture Technology, Bielefeld University, for his willingness to examine this thesis. It was a pleasure for me to work with him on courses of molecular biotechnology. His friendly nature made him a dear colleague. In addition, his motivation and enthusiasm has brough a breath of fresh air into the department of biotechnology. I wish him all the best here in Bielefeld. He certainly has the capabilities to make a difference.

Results from scientific research are not earned by a single person but are rather a team effort. Several people have worked with me and their contribution is gratefully acknowledged. Kirsten Leiwig has accompanied me from the beginning. As a laboratory assistant she performed her work with outstanding diligence. I wish her all the best during her maternity leave and hope that she and her family are always well.

Dipl. Ing. Nadine Streitner worked for her PhD under my supervision for the last three years. Her contribution as well as the very pleasant professional relation is also deeply acknowledged. It was always delightful to work with her.

Furthermore, several diploma students have been supervised. Working with them was a pleasure. Therefore, I would like to thank Josef Schulze-Horsel, Lars Christian Keßler,

Acknowledgdments

Dennis Lindau, Anja Hasche, Hendrik Kortmann, Antonia Bär, Xiaohong Huang, Tanja Vollmer, Alexander Kellner, Martin Block, Ilka Husemann, Jürgen Abel, Manfred Schrewe, Philipp Claar, Petra Bellwied, Jan Fischer, and Dominik Cholewa. Additionally, I would like to thank Kirsten Tschapalda and Nils-Christian Lübke for their contribution during their B.Sc. theses. Kirsten Tschapalda also deserves special gratitude for working as a student assistant on the DFG funded nucleic acid extraction project.

Furthermore, I would like to thank all the students that I have encountered during my teaching activities and their undergraduate projects. I hope that I was able to spark their interest for bioprocessing and to refer some of my knowledge to them.

Last but not least, I would like to thank all the members of the Chair of Fermentation Engineering, especially Dipl. Ing. Thomas Schäffer for keeping all the equipment running and his dedication to solving all kinds of technical problems and Petra Widawka for her work in the laboratory.

1 Summary

Plasmid DNA is currently used in gene therapy and genetic vaccination as a vector system for the delivery of therapeutic genes. Clinical trials as well as future therapeutics demand large amounts of high quality plasmid DNA that fulfils the specifications set by regulatory authorities. This thesis describes the development, analysis, and evaluation of pharmaceutical plasmid DNA production processes comprising cultivation, product isolation, and purification as well as stability assessment during storage and application. Cultivations on defined media have been analyzed and compared to state of the art cultivations on semidefined medium. The influence of amino acid supplementation as well as the effect of the physiological conditions of the inoculation culture on growth and product formation have been determined. In this way, batch cultivation processes utilizing glycerol based defined media could be established having yield coefficients $Y_{X/S}$ and product selectivities $S_{P/X}$ comparable to or exceeding the values obtained with a semidefined medium. Additional proteome analysis indicated a stringent response that could influence plasmid replication. A semi continuous alkaline lysis was combined with froth flotation for large scale product isolation. The procedure was able to produce a highly clear lysate that could directly be applied to subsequent purification. This process was compared to other modes of operation with respect to product formation, contamination with chromosomal DNA, and plasmid form distribution. For the purification of plasmids, DNA-binding proteins were analyzed as potential affinity ligands. In addition, a recombinant RNase has been produced and its capability for RNA depletion could successfully be demonstrated. The partitioning of nucleic acids in reverse micellar two-phase systems was examined and used to develop an extraction process for plasmid purification which could be integrated into different purification schemes that allowed the complete depletion of all contaminants. Finally, the stability of purified plasmid DNA during long term storage and its implication on the effectivity after gene transfer has been investigated.

2 Introduction

Biotechnology products have been used by humanity for millenia. During the last century, the industrialization of bioproduction has led to significant changes in our life. This was made possible by a profound understanding of biological processes in combination with engineering that increased the scientific knowledge drastically during the recent decades.

Milestones in biotechnology were certainly the industrial scale production of antibiotics and the production of therapeutic proteins like insulin that raised the quality of life in the industrial nations. At the beginning of the 21st century, biotechnology is considered to be one of the key technologies to solve some of the major problems of our society. Renewable resources should be used for manufacturing and energy production. The latter topic has gained considerable attention during the recent years, especially with increasing energy costs and the prospect of limited oil resources on a long term perspective.

In the future, biotechnology research will also focus on the development of highly scalable and sustainable production processes. When looking at the demographic development, resources will represent the limitations for this processes, and scientists are therefore challenged to make processes and products available to the majority of humanity.

Several drug candidates have evolved from biomedical research and are now produced at an industrial scale. Scientists continuously increase the number of targets as well as the technologies for their production. Among these new biomedical treatments, genetic medicine has gained considerable attention during the recent years and the hope to cure several diseases are pinned on it.

Genetic medicine has been discussed in principle in the 1970s already. The development of genetic tools made first experiments in this new field of medical treatment possible. At first, monogenic diseases and incurable infections were the primary targets, but later on cancer and cardiovascular diseases moved more and more into the focus of gene medicine. Several methods have been used for the transfer of therapeutic sequences into the patient since then and gene delivery is continuously optimized.

Plasmid DNA is one of the non viral delivery vectors that has been examined since the early days of gene medicine. This nucleic acid is commonly found in bacteria and other microorganisms. In there, it usually replicates independently and codes for genes that give the host a selective advantage, *e.g.* antibiotic resistance or auxotrophic complements. In genetic engineering plasmid DNA has become a valuable tool for the introduction of genes into microorganisms and genetic manipulation. Thus, its application as a vector system for therapeutic genes in gene medicine seemed only obvious.

In parallel to the development of therapies for several indications, processes for large scale plasmid manufacturing have been designed. These methods usually comprise cultivation of plasmid replicating bacteria, commonly *Escherichia coli*, alkaline lysis combined with RNase A treatment, and subsequent purification of the plasmid DNA by anion exchange chromatography. Cultivation strategies that were used for recombinant protein production have been adapted and downstream processing was based on procedures that were applied for the isolation of cloning vectors. It was soon realized that these methods did not meet all expectations. Cultivation processes were suffering from low product selectivity, especially at larger scale and when using defined media. Downstream processing could not be scaled up easily and the desired quality has not been achieved.

This situation can be considered as the starting point of the research presented in this thesis. As a first step, cultivation processes had to be analyzed and developed further with respect to product selectivity. Guidelines set by regulatory authorities had to be taken into account. Cell disruption is the second step in the production process. Because alkaline lysis can also be considered as an initial purification, this method was chosen and scaled up so that large amounts of plasmid harboring biomass could easily be disrupted. The capturing step was considered to be the most challenging part of the whole downstream processing. Several process options were developed and analyzed for their applicability in large scale plasmid manufacturing in this thesis. These options were the development and application of a recombinant RNase, extraction using reverse micellar two-phase systems, and affinity purification using DNA binding proteins. Further cleaning was accomplished by state of the art methods, like hydrophobic interaction or anion exchange chromatography. In the latter case some basic research was also performed on stationary phase design by grafting monolithic columns with methacrylate polymers in

order to enhance their dynamic capacity (not presented in this thesis). The development and examination of cultivation processes was mainly covered by the diploma thesis of Antonia Bär (Bär, 2005) and Jan Fischer (Fischer, 2008). The scale up of the alkaline lysis procedure used for cell disruption has partially been published already (Voss, 2007; Voss *et al.*, 2005) and was further examined by Kirsten Tschapalda in her B.Sc. thesis (Tschapalda, 2008). The recombinant RNase was developed by Dennis Lindau and its application for RNA digestion was succesfully demonstrated (Lindau, 2004; Voss *et al.*, 2006). Basic investigations with respect to protein-DNA interaction have been conducted by Anja Hasche (Hasche, 2004; Hasche and Voß, 2005) utilizing the well known Lac repressor protein. In parallel, Hendrik Kortmann analyzed the DNA binding properties of the replication initiator protein RepU (Kortmann, 2005) which was continued in the diploma thesis of Jürgen Abel (Abel, 2006) who immobilized the protein on monolithic disks, and Petra Bellwied (Bellwied, 2008) examining the affinity chromatography step with respect to binding capacity and selectivity. The partitioning of nucleic acids in reverse micellar two-phase systems was examined by Nadine Streitner (Streitner *et al.*, 2007). Furthermore, an extraction method was developed from these results and integrated into highly scalable and robust downstream processes (Streitner *et al.*, 2008). Plasmid DNA stability has been published in two articles (Walther *et al.*, 2002; Walther *et al.*, 2003). For the sake of completeness, it should be mentioned that some research did not find a place in this thesis. The results of these students are, however, gratefully acknowledged and a complete list of the supervised theses is presented in the appendix section.

Following this short introduction, the theoretical background will present a more detailed insight into the field of plasmid DNA and its production for pharmaceutical application. The intention of that chapter is not to give a complete review of the different aspects but rather a comprehensive background information based on key papers published in the recent years. Subsequently, the research results are presented and discussed thoroughly followed by a final discussion, aspects of current research as well as future directions.

3 Theoretical Background

Bioproduction using cell cultures or microorganisms usually follows a general scheme that is presented in Figure 1. The cultivation process is followed by a solid liquid separation process to separate the production host from the culture medium. The next step depends on the localization of the product. In one case, the product is secreted into the medium and has to be isolated from the culture liquid. In the second case, the product remained inside the cells and has to be isolated by cell disruption first before subsequent purification can be applied. The purification procedure usually attempts to capture the product. Ideally, major impuritues are depleted at this stage already and the product is concentrated substantially, thus making further steps less tedious and time consuming. The rest of the contaminants are removed by purification and polishing steps and the product is formulated in a suitable form with respect to its application. For purification, several unit operations commonly used in chemical engineering can be applied for bioproduction. The application of each unit operation as well as the number of purification steps is determined by the chemical and physical properties of the product and the desired purity. The latter one depends on the application of the product.

Plasmid manufacturing for pharmaceutical application comprises three basic steps. First, the plasmid replicating host organism, *Escherichia coli*, has to be cultivated. As the second step the cells have to be disrupted to liberate the DNA and subsequently the plasmids have to be purified being the third step of the manufacturing process. Basically, this simplified production scheme differs in no way from the methods used for the isolation of plasmid DNA as cloning vectors. The main differences originate from the requirements set by regulatory authorities, desired quality, and production scale.

For the development of a successful production process, a profound understanding of several disciplines is necessary ranging from biochemistry and molecular biology over biochemical engineering to physical chemistry. The successful development should also be accompanied by sophisticated analytical techniques documenting the process and assuring the quality of the final product.

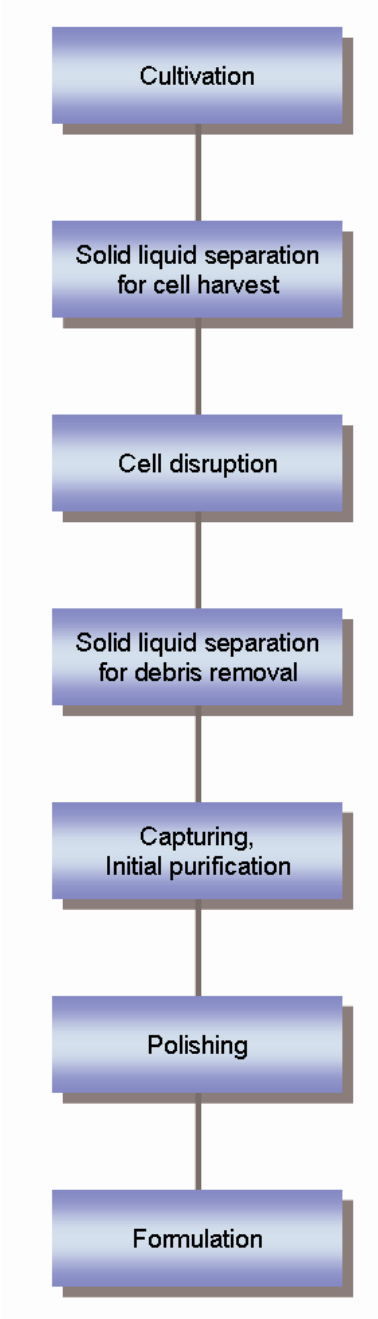


Figure 1 General production scheme for biomanufacturing of intracellular products.

In the following, a comprehensive introduction is given into the different aspects associated with pharmaceutical plasmid DNA production. In addition, this chapter outlines how this research also addresses areas that are not only an issue for plasmid DNA but also hot topics for biotechnological research in general.

3.1 Plasmids

For biopharmaceutical plasmid production high copy number vectors are preferred. When using these systems as carriers for the therapeutic sequence, comparably high product concentrations are observed. However, plasmid concentration in the host organism is controlled by several mechanism of the replication control system. It is necessary to understand the mechanism of plasmid replication in order to evaluate the effects of culture conditions on product formation during reactor cultivations.

3.1.1 Plasmid Copy Number and Plasmid Stability

Plasmid replication in *Escherichia coli* usually follows the theta mechanism. A well characterized family with this mode of replication is formed by the ColE1 derived plasmids. The naturally occuring ColE1 plasmid codes for colicin, a protein with antibiotic properties that gives the host organism a selective advantage over other microorganisms. Several well known cloning vectors like pBR322 (Bolivar, 1978) or the pUC family (Lin-Chao *et al.*, 1992) are derived from ColE1.

These plasmids are replicated under relaxed control independant from the host's chromosome and protein biosynthesis. They usually appear as low copy (up to 20 molecules per cell) or high copy (100 – 1000 molecules per cell) number plasmids. The replication of ColE1 plasmids begins with the transcription of RNA II by RNA polymerase that works as a preprimer for plasmid replication. The hybridization of RNA II with the template DNA is partially inhibited by an 108 nucleotide antisense transcript called RNA I. The formation of the RNA II/RNA I hybrid is further stabilized by the Rom protein (Rom: RNA one modulator) (Atlung *et al.*, 1999). Removal of the *rom* gene from pBR322 increased plasmid copy number by a factor of 2 (Twigg and Sherratt, 1980). The pUC plasmids also lack the rom gene, but in addition they have a G-A point mutation in RNA II, inhibiting the formation of the RNA II/RNA I duplex. This effect is sensitive to temperature. While at 30 °C no change in plasmid copy number is observed, a considerably higher value is measured at 42 °C (Lin-Chao *et al.*, 1992). Further replication follows the theta mechanism (Summers, 1996).

The structural instability of plasmid DNA is caused by mutations and leads to inefficient gene expression. A more common issue in plasmid production is the segregational

instability of plasmid DNA. The constitutive expression of plasmid coded proteins is a metabolic burden for the host which results in slower growth rates of plasmid bearing cells. Insufficient inheritance can result in plasmid free cells with comparably faster growth rates. As a consequence, a cultivation can be overgrown with plasmid free cells which will evidently decrease the average plasmid copy number of the whole population and therefore result in decreased product concentrations. This effect can be compensated by the addition of antibiotics, but it is usually avoided in plasmid manufacturing due to economic and mainly regulatory aspects. Complementation of host strain auxotrophies is another strategy that has been described (Kumar *et al.*, 1991). Sufficient replication and plasmid inheritance can also be achieved by controlled growth on optimized media (O'Kennedy and Patching, 1997). Earlier research indicated a connection between the specific growth rate and plasmid copy number (Schmidt, 1998; Voß, 2001; Voss *et al.*, 2003; 2004). However, a generally accepted rule for the effect of cultivation conditions on plasmid replication has not yet been formulated.

3.1.2 Plasmid Forms

Plasmid DNA has a circular double helix. Because of this circular structure, it can exist in different topological forms (plasmid isoforms) that are shown in Figure 2. Inside the cells plasmid DNA exists in a negatively supercoiled form, also called closed covalently circular (ccc) form. Single strand breaks by enzymatic activity or due to mechanical stress result in the formation of the open circular (oc) form. This relaxed isoform cannot be distinguished from the relaxed form with intact strands by simple electrophoretic analysis. Restriction enzymes cut the DNA double strand at defined recognition sites. Their activity results in the formation of a linear form. Multimeric plasmid forms, called concatemers are caused by homologous recombination. They are a covalently connected sequence of one or more plasmid molecules. Although multimeric forms can be produced in *E. coli* (Voss *et al.*, 2003), no serious application or advantage in gene medicine has been found for these plasmid molecules yet.

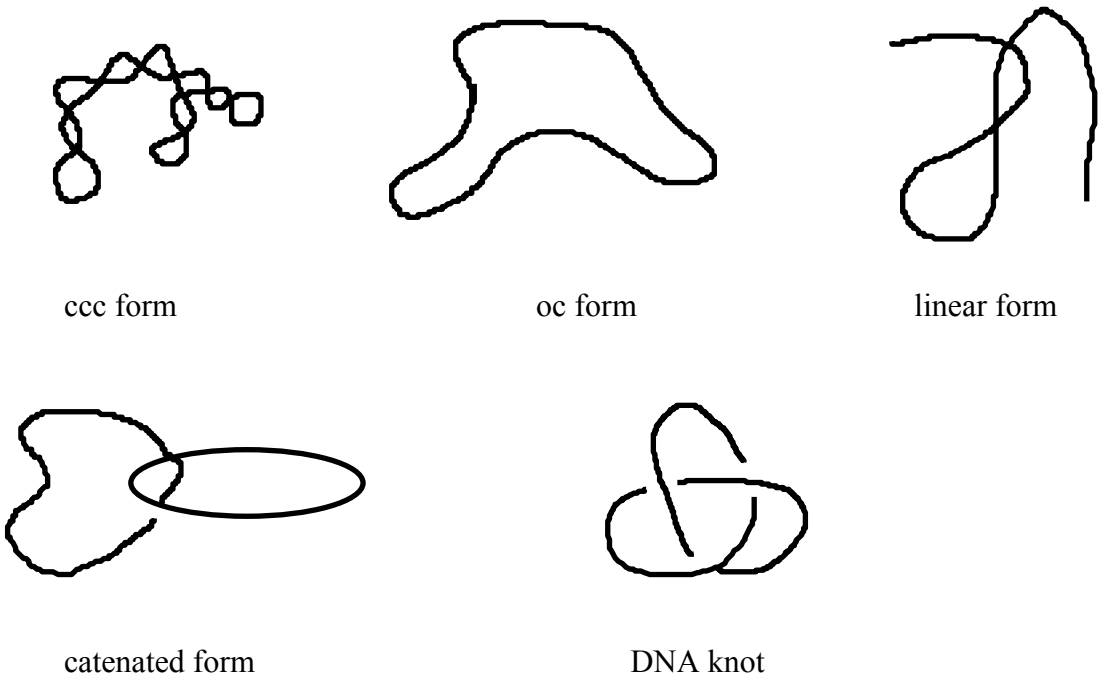


Figure 2 Different plasmid DNA topologies (Voß, 2001).

Catenated forms and DNA knots are usually observed as products of certain topoisomerases and other enzymes *in vivo* (Kusano *et al.*, 1989).

The relative amount of plasmid DNA isoforms can be determined by agarose gel electrophoresis (AGE). In a simple manner, migration depends on the shape of the nucleic acids. The more compact ccc forms migrate much faster than the larger oc forms. However, this method is not able to completely resolve the open circular monomer from the ccc dimer forms for larger plasmids (> 4.6 kb) (Schmidt *et al.*, 1999b). Additionally, densitometric analysis can be afflicted with larger errors giving inaccurate results. Figure 3 shows the analysis of different pUC19 samples (2.7 kb) by agarose gel electrophoresis. The two prominent forms in the untreated samples are the ccc monomer and the ccc dimer (lane 2). Both forms could be identified by comparison with results obtained from electron microscopy (Schmidt, 1998).

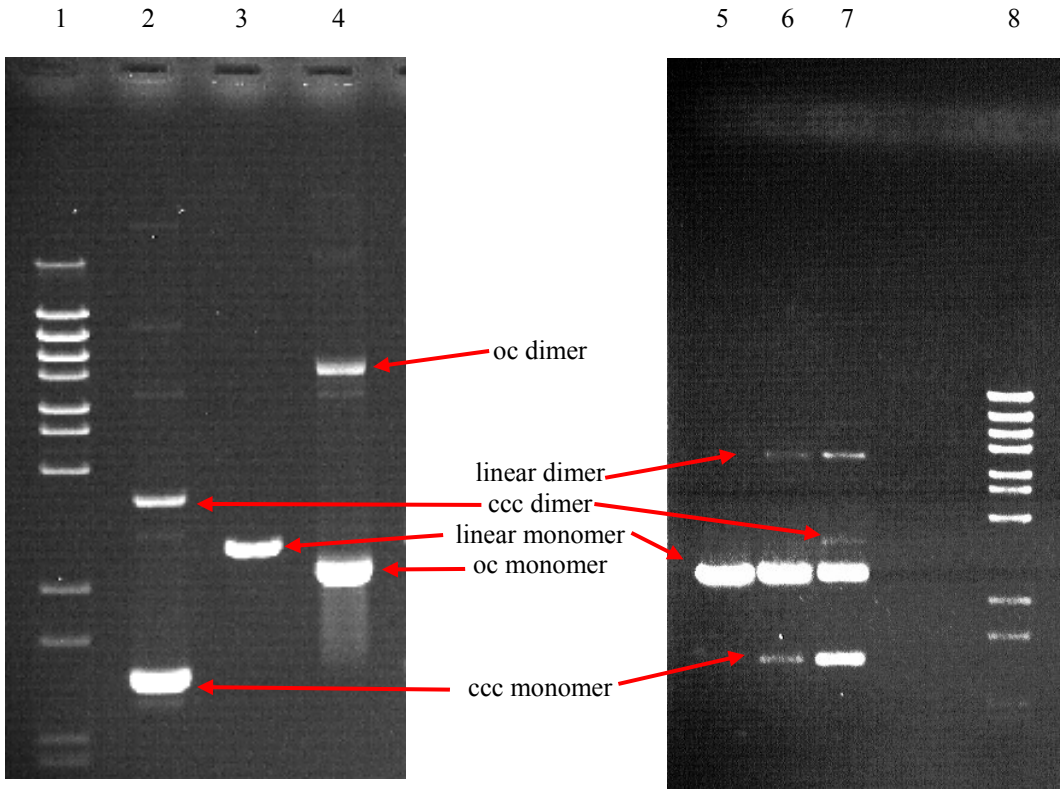


Figure 3 Agarose gel electrophoresis of pUC19 samples. Lanes 1, 8: DNA size marker (λ-DNA, BstEII cut). Lane 2: untreated sample. Lane 3: EcoRI digested sample. Lane 4: UV irradiated sample. Lane 5: partial digestion with 1 U EcoRI. Lane 6: partial digestion with 0.1 U EcoRI. Lane 7: partial digestion with 0.01 U EcoRI (Schmidt, 1998).

Digestion with the restriction enzyme EcoRI resulted in the formation of the linear monomer (lane 3). Irradiation with UV light introduced single strand breaks and oc forms were formed. In lane 4, the oc monomer and the oc dimer are the predominant forms. To verify if a linear dimer form could be present in the samples, partial digestions with different enzyme concentrations were performed. At high enzyme concentrations only the linear form is observed (lane 5) that was also identified in the sample in lane 3. At lower enzyme concentrations, an incomplete digestion was observable because ccc monomer form is still detectable and a new form is visible (lane 6) which could be identified as linear dimer. Lane 7 shows an undigested fraction of the ccc dimer form in addition to the other forms. Schmidt deduced the following migration order in agarose gel electrophoresis (fast first): ccc monomer > oc monomer > linear

monomer > ccc dimer > linear dimer > oc dimer. In conclusion, the monomer forms migrate faster than the dimer forms (Schmidt, 1998; Schmidt *et al.*, 1999b).

Because of the disadvantages of agarose gel electrophoresis, an analytical method based on capillary gel electrophoresis (CGE) with laser induced fluorescence (LIF) detection has been developed which could resolve the different topologies more efficiently (Schmidt *et al.*, 1999b).

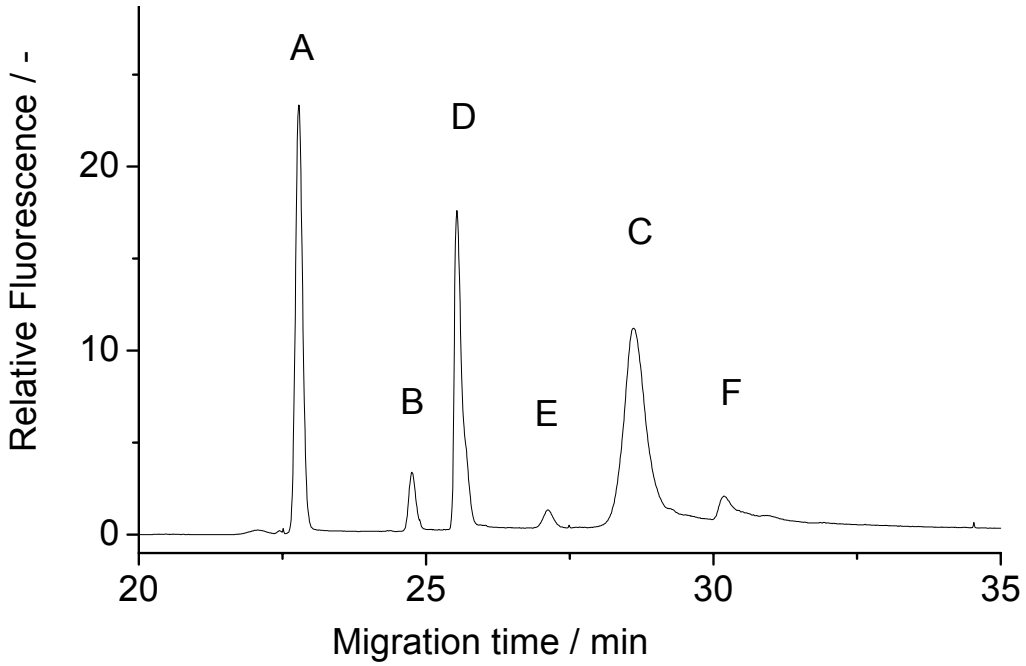


Figure 4 Capillary gel electrophoresis of a plasmid pUC19 mixture containing ccc monomer (A), ccc dimer (B), oc monomer (C), linear monomer (D), linear dimer (E), and oc dimer (F) (Schmidt, 1998).

Figure 4 shows the CGE analysis of a pUC19 mixture containing the six different plasmid forms that were already described in the agarose gel in Figure 3. By comparison and spiking, the peaks in the electopherogram could be identified and the following migration order could be deduced for CGE separation: ccc monomer > ccc dimer > linear monomer > linear dimer > oc monomer > oc dimer. Thus, the ccc forms migrate in front of linear, and oc forms. The results did not take higher multimeric forms (trimers, tetramers, *etc.*) into account. In case of pharmaceutical plasmid production higher mul-

timers are not observed in the preparation and a separation from other forms is therefore not necessary. A more detailed analysis of such samples was performed by Voß (2001), utilizing linearized polyacrylamide as a separation medium for capillary gel electrophoresis.

Nevertheless, CGE analysis has evolved as a valuable tool for the analyis of plasmid form distribution in process development (Streitner *et al.*, 2007), quality control of plasmid DNA, and for stability analysis (Walther *et al.*, 2002; Walther *et al.*, 2003) which will be outlined in this thesis.

3.2 Gene Therapy

In gene medicine therapeutic genetic elements are brought into the cells to treat a genetic malfunction of the organism. These can be monogenic diseases or multifactorial disorders. In principle genes can be introduced into germline or somatic cells, but the first case is prevented by the German Embryo Protection Act.

Gene transfer into somatic cells leads to the synthesis of therapeutic proteins by the body's own cellular mechanism. In this way an optimal protein expression with respect to posttranslational modification is expected directly at the physiological place of the disorder inside the organism (Cichutek, 2001).

The first indications to be treated by gene therapy were monogenic diseases like cystic fibrosis, adenosine desaminase (ADA) deficiency, or gaucher disease. However, soon the main disorders of our civilization, like cancer and cardiovascular diseases, became the primary targets of gene therapy efforts.

As shown in Figure 5, 67 % of the more than 1300 clinical trials were dealing with cancer, while only 8 % are focussed on monogenic diseases. A comparable part of the trials is dealing with the treatment of cardiovascular (9 %) and infectious diseases (6 %). The focus on cancer can be explained by economic interests. In case of infectious diseases, preventive and curative vaccination against infections lacking a classic therapy, *i.e.* HIV (Boyer *et al.*, 2000), hepatitis C (Duenas-Carrera *et al.*, 2004), *etc.*, is attempted. Here, genetic vaccination is achieved by the introduction of antigene coding sequences into the organism resulting in an immune response.

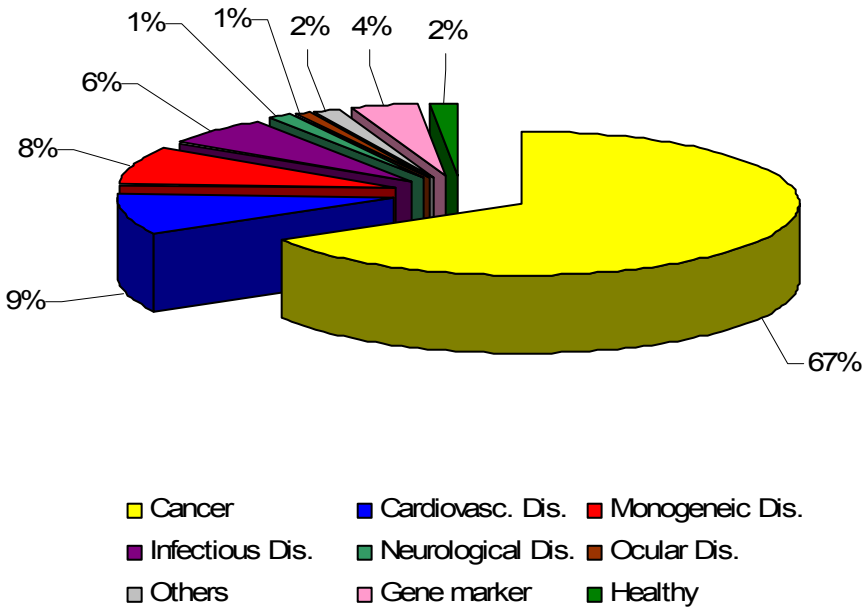


Figure 5 Indications treated in gene therapy trials in 2007 (**http://www.wiley.co.uk/genetherapy/clinical/**).

This principle has first been introduced by Wolff *et al.* (1990) by the *in vivo* expression of a reporter protein after injection of plasmid DNA into a mouse muscle. Further research in this field (Danko and Wolff, 1994; Wolff *et al.*, 1992) increased both the interest in genetic vaccination and also in plasmid DNA as a vector system.

With respect to the vector systems that can be used for the transfer of the genetic sequences, two different approaches have to be distinguished, *i.e.* viral and non viral vector systems. As shown in Figure 6, most clinical trials work with different viral vectors while only 18 % utilized plasmid DNA.

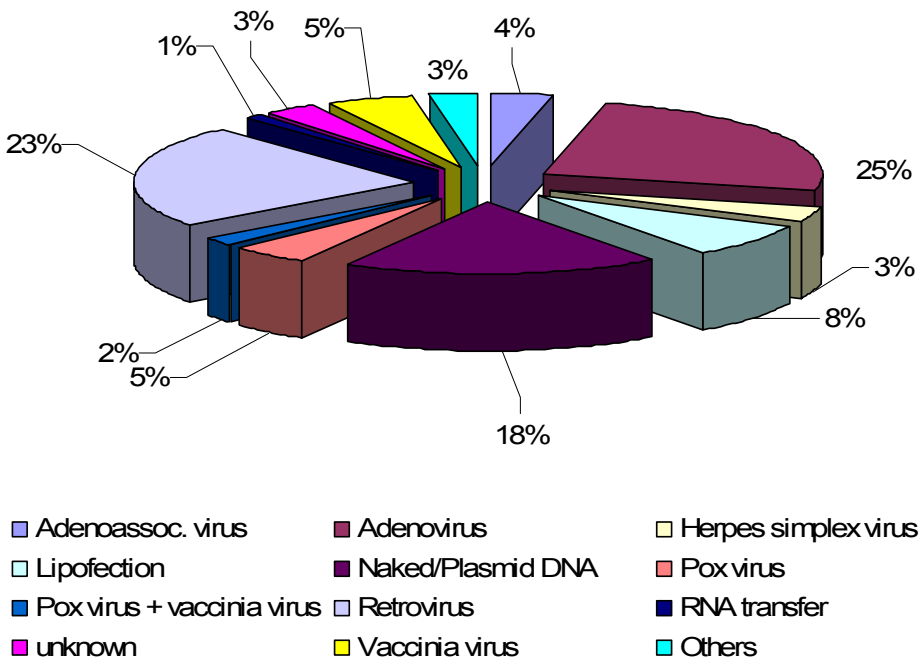


Figure 6 Vector systems used in gene therapy clinical trials in 2007 (http://www.wiley.co.uk/genetherapy/clinical/).

The advantages of viral systems are their more efficient cell targeting properties in comparison to non viral vectors. On the contrary, viral vector systems are difficult to produce and their application is afflicted with several safety concerns. An already existing immunity against the virus can result in a failure of the treatment or even worse in an immunological shock with life threatening proportions as shown in the case of Jesse Gelsinger (Stolberg, 1999). Another safety risk associated with viral systems is the integration of viral genetic information into the host chromosome. As a result of this integration oncogenes can be activated leading to cancer like symptoms (Check, 2002). No safety concerns are associated with non viral delivery systems like plasmid DNA and in comparison to viral vector systems their manufacturing is relatively simple. However, one crucial disadvantage is their poor cell targeting capability. Therefore, plasmid DNA became a more attractive vector for genetic vaccination which has gained considerable interest not only in human, but also in veterinary medicine because the vaccination of large populations even with multiple antigens became possible with a single treatment. In case of veterinary medicine, some therapeutics have already been approved by the

US Food and Drug Administration (FDA), *e.g.* a vaccine for horses against the West Nile virus (Powell, 2004).

Plasmid vectors that are used for gene therapy or genetic vaccination are in principle the same vectors that are used in molecular cloning. In addition to the genetic elements necessary for replication in *E. coli* (replication origin, selection marker) they carry a therapeutic protein sequence in combination with an eukaryotic promoter facilitating the expression of the therapeutic genes in treated mammals. It should be noted that the genetic sequences for stable replication in *E. coli* were found to be interfering with gene expression in the transfected animal cells (Chen *et al.*, 2003). This led to the development of mini circle plasmids (Chen *et al.*, 2005) prepared by a ΦC31 integrase-mediated intramolecular recombination technology that are devoid of any bacterial sequences. Although these vectors could be synthesized by this technology in principle, their large scale manufacturing by sophisticated cultivation techniques is still a dream of the future.

3.3 Escherichia coli as a Production Host

Escherichia coli is used for pharmaceutical plasmid production because this organism is well characterized and replicates plasmid DNA to high copy numbers. A basic introduction into the metabolism of *E. coli* and the factors influencing the physiological state of the organism precedes the description of cultivation processes that have been used for pharmaceutical plasmid DNA production.

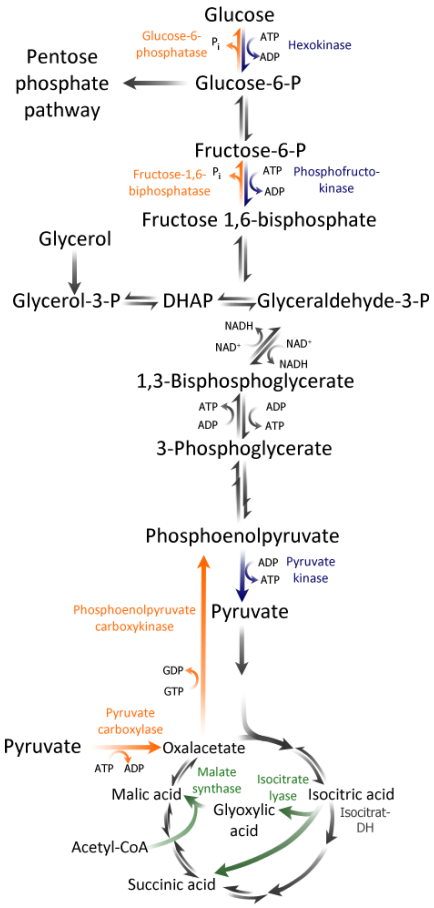
Escherichia coli needs several nutrients for growth. Next to a carbon source, nitrogen, phosphor, and sulfur have to be supplied as macro elements while manganese, zinc, nickel, cobalt, copper, sodium, and selenium are only necessary in trace amounts.

3.3.1 Carbon Sources and Energy Generation

The carbon source is used for energy generation (catabolism) by the microorganism. Carbon is oxidized by several reactions resulting in energy output and the formation of molecular precursors for the synthesis of all the molecules inside the organism (anabolism). Products of the catabolism are ATP that serves as an energy source, carbon dioxide, and water.

Several sugars, polyols, and carboxylates can be utilized by *E. coli*, *e.g.* D-glucose, maltose, raffinose, galactose, fructose, D-mannose, D-sorbose, glycerol, D-mannitol, and D-sorbitol (Lin, 1996). Proteinogenic amino acids can also be used as a carbon source. They are taken up by different transporter systems and after desamination the residual carbon framework enters the citric acid cycle.

3.3.2 Glycerol as Carbon Source



In the research presented here, glycerol is used as the main carbon source because the formation of inhibitory byproducts is avoided. Glycerol diffuses through the cell wall into the periplasm and is transported by facilitated diffusion by the glpF membrane protein into the cytoplasm (Sweet *et al.*, 1990) where it is phosphorylated by a MgATP dependent glycerokinase resulting in glycerol-3-phosphate (glycerol-3-P). Subsequently, it gets metaboblized to dihydroxyacetone phosphate (DHAP) (Figure 7) which can either be oxidized to pyruvate and further to acetyl-CoA that enters the citric acid cycle or, alternatively, it can be used for the generation of glucose via the pentose phosphate pathway. Both pathways cannot be used in parallel by *E. coli* in all steps.

Figure 7 Integration of glycerol in metabolic pathways.

When using glycerol as a carbon source, gluconeogenesis is necessary to build β-D-glucose phosphate and fructose 6-phosphate for the *de novo* synthesis of nucleotides, coenzymes, and other essential metabolites.

3.3.3 Nitrogen Sources and Nitrogen Metabolism

Nitrogen is an essential element for the growth of microorganisms because it appears in amino acids, purines, and pyrimidines. Several organic compounds, *e.g.* amino acids, as well as inorganic compounds, mainly ammonia or ammonium salts, can be used by *E. coli* as nitrogen source. Ammonium compounds are transported into the cell by the ammonium transporter Amt. Ammonium is in chemical equilibrium with ammonia that can diffuse into the cell as an uncharged molecule. At low concentrations, ammonium is bound to the Amt transporter and deprotonated, thus entering the cytoplasm as uncharged ammonia.

In case of excess nitrogen ammonia is deprotonated and assimilated by the glutamate dehydrogenase (GDH) as shown in Figure 8.

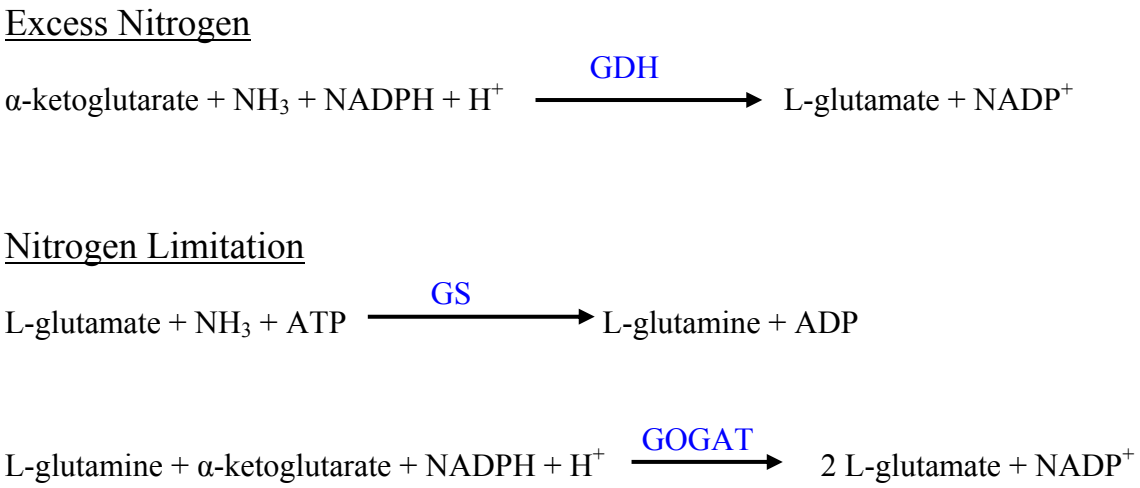


Figure 8 Nitrogen assimilation in *Escherichia coli*.

At low nitrogen concentrations, glutamine synthetase (GS) adds ammonia to L-glutamate by the consumption of ATP. The resulting L-glutamine reacts with α-ketoglutarate to L-glutamate under the activity of glutamate synthase (GOGAT)

3.3.4 Biosynthesis of Amino Acids and Nucleotides

Escherichia coli is capable to synthesize all 21 amino acids *de novo* using intermediates from the citric acid cycle, the pentose phosphate pathway and glycolysis. Nitrogen en-

ters these reactions as described in the previous section. Additionally, transaminases can transfer the nitrogen from one amino acid to the other. Many amino acids are synthesized via the same metabolic pathways and can be classified in different families according to their precursors. These precursors and their origin in the metabolism are shown in Figure 9.

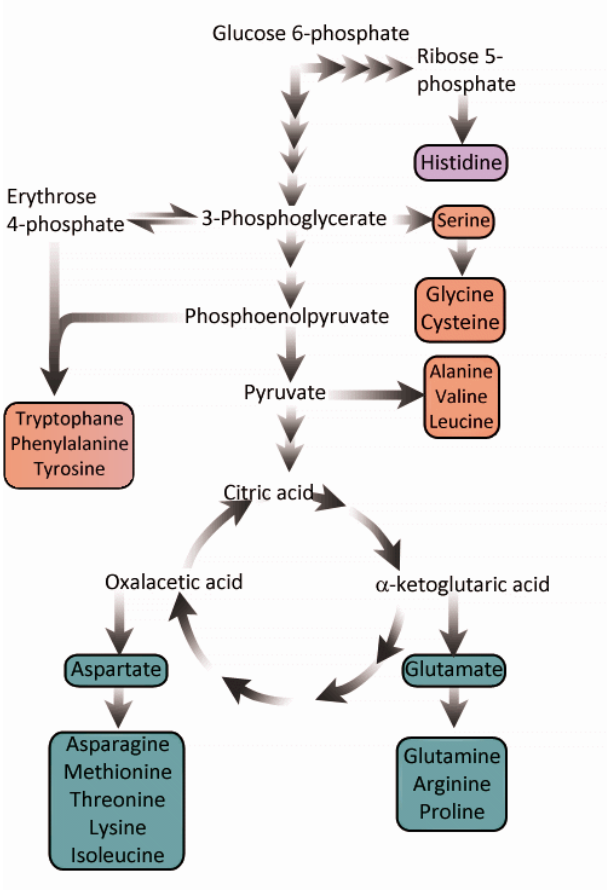


Figure 9 Amino acid precursors in the central metabolism. Precursors originating from the pentose phosphate pathway are marked purple, the ones from glycolysis red, and the ones from the citric acid cylce are in green.

Nucleotides can either be produced by *de novo* synthesis or by salvage pathways. *De novo* synthesis starts with phosphoribosyl pyrophosphate (PRPP) that is synthesized from ribose phosphate via the pentose phosphate pathway. In case of purines, an amino group from glutamine is bound to the C1 atom of PRPP giving the highly unstable

phosphoribosyl amine. Further reaction lead to the generation of inosinic acid (IMP) which can be converted to adenosine (AMP) or guanosine monophosphate (GMP).

Pyrimidines are synthesized from carbamoyl phosphate and aspartate by aspartate transcarbamoylases (ATCase) giving orotic acid. This pyrimidin ring is coupled with ribose 5-phosphate from PRPP giving finally UTP and CTP

3.4 Factors Influencing the Physiological State of Escherichia coli

Escherichia coli has to react to changes in the environment in order to survive. A complex network of regulatory components enables the microorganism to adapt to changes quickly. These components are DNA, mRNA, siRNA and special proteins interacting with nucleic acids. During growth on defined medium, important components are missing and the metabolism of the cell has to adapt.

Transcription under physiological stress is regulated by histon like proteins, sigma factors, and two component systems. In prokaryotic microorganisms sigma factors are necessary to initiate transcription. These molecules bind to the Pribnow box of the promoter and increase the probability of polymerase binding (Neidhardt, 1996).

Seven sigma factors are found in *E. coli* that are induced due to environmental changes. Under normal growth, σ^{70} is expressed solely facilitating the transcription of genes for cell growth. Chaperons and heat shock proteins are induced under temperature stress by σ^{32}. The gene regulation for the absorption of iron citrate is carried out by σ^{19} (Visca *et al.*, 2002), while σ^{38} (σ^{S}) is responsible for the general stress response. Sigma factor σ^{54} regulates the genes for nitrogen metabolism and substrate specific transporter systems for alternative energy sources, σ^{28} regulates chemotaxis genes (Helmann, 1991), and σ^{E} is induced due to exocytoplasmic stress.

Alarmones like ppGpp and cAMP/CRP are small effector molecules that can constitute a new stationary state in gene expression due to changes in the environmental conditions (Wick and Egli, 2004)

When different substrates are available to *E. coli*, the ones that can be metabolized most quickly are preferred. This effect is known as catabolite repression. The cAMP/CRP system regulates carbon uptake into the cell. When the concentration of quickly metabolized carbon sources is limited, transporter systems for alternative carbon sources are

expressed (Chang *et al.*, 2002). Further limitation of the carbon source results in a general stress response by σ^S (Loewen and Hengge-Aronis, 1994) initiating the stationary growth phase. Cra is a catabolic regulator protein controlling carbon flow under carbon limitation. Both systems are reviewed (Harman, 2001; Pastan and Perlman, 1970) and will not be discussed in details here.

The stringent response triggers a mechanism inside the cell that utilizes the existing resources more efficiently resulting in a changed anabolism and protein biosynthesis. As a consequence, the concentrations of the involved molecules are reduced. Limited concentrations of nutrients, oxidative stress, ethanol, and an increased osmolarity can trigger the stringent response as described by Borek *et al.* (1956) and Neidhardt (1996). These stress factors lead to an increased concentration of (p)ppGpp that changes the cell's anabolism (Cashel *et al.*, 1996)

3.5 Cultivation Processes

For the cultivation of microorganisms several systems and strategies have been developed. Research in this area is concerned with bioreactor design, modes of operation, *i.e.* batch, fed batch, or continuous process, aeration, *etc*.. In addition, proteomic and metabolomic analyses have been applied to understand and further optimize the cultivation parameters and in this way increase the productivity of the processes (Hiller *et al.*, 2007). Next to this, the development of small scale parallel cultivation systems made a more systematic analysis of different parameters possible (Weuster-Botz *et al.*, 2005) resulting in a rather rational design with respect to media compositions and other cultivation parameters (Weuster-Botz, 2000).

Appropriate conditions for bioreactor cultivation largely depend on the producing organism and the factors influencing product formation inside the cell. Thus, a general formulation is certainly not possible, while a more detailed insight into this topic would exceed the scope of this thesis. Therefore, some general remarks should be made concerning the cultivation of *E. coli* followed by a presentation of the cultivation processes used for pharmaceutical plasmid production.

The nutrient requirements as well as several factors influencing the growth and product formation during bioreactor cultivation have already been outline in the preceding sec-

tion. *Escherichia coli* is a facultative anaerobic microorganism. Oxygen is preferred as a terminal electron acceptor, but other molecules like nitrate and nitrite are also utilized (Schulze-Horsel, 2003). The critical concentration of oxygen for *E. coli* is 0.25 mg L^{-1} (Bailey and Ollis, 1986). Values below will result in reduced growth. The oxygen uptake rate (OUR) is defined as the product of the specific oxygen demand q_{O_2} and the biomass concentration X. The specific oxygen demand is depending on the host strain and the culture conditions applied.

$$OUR = q_{O_2} \cdot X \qquad \text{(Eq. 1)}$$

Because oxygen shows a concentration of 9.4 mg L^{-1} in water at 20 °C (Mutzall, 1993), a continuous aeration during the whole cultivation process is necessary. Oxygen transport can be described by a film diffusion process (Nielsen and Villadsen, 1994). The oxygen transfer rate OTR is defined as shown in equation 2 with $k_L a$ as the volumetric oxygen transport coefficient and c_l^* as the oxygen equilibrium concentration and c_l as the actual oxygen concentration in the liquid.

$$OTR = k_L a \cdot \left(c_l^* - c_l\right) \qquad \text{(Eq. 2)}$$

The equilibrium concentration depends on the total gas pressure and the oxygen fraction of the gas. The specific interface is correlated with the diameter of the gas bubbles. Increasing gas pressure, oxygen fraction in the gas, or the volumetric flow rate can increase the oxygen transfer rate. Furthermore, an increased application of engergy by using an elevated stirrer frequency or a better stirrer geometry, will change the film thickness as well as the diameter of the gas bubbles which will also result in a higher OTR.

Growth is described by an increase in the biomass concentration as shown in equation 3. The growth rate r_X is proportional to the biomass concentration X and the proportionality factor μ is called the specific growth rate.

$$r_X = \frac{dX}{dt} = \mu \cdot X \qquad \text{(Eq. 3)}$$

The specific growth rate remains constant at a maximum value as long as no nutrient limitations are encountered. On nutrient limitation, μ is falling and reaches a value of

$\mu = 0\ h^{-1}$ when the substrate is completely utilized. Growth is suspended and the stationary phase is reached. The correlation between the specific growth rate and the concentration of a limiting susbtrate is described by the Monod equation (equation 4).

$$\mu = \mu_{max} \frac{S}{S + K_S} \qquad \text{(Eq. 4)}$$

The parameter K_S is called the Monod constant and is specific for organisms and substrates. K_S represents the subtrate concentration where $\mu = 0.5\ \mu_{max}$. When S is exceeding K_S, $\mu \approx \mu_{max}$ and a zero order kinetic with respect to the substrate concentration S is observed. When S is very low ($S << K_S$) equation 4 is valid describing a first order kinetic with respect to S. In addition, K_S describes the quality of the substrate for the microorganism. When low values of K_S are observed, the substrate is utilized very efficiently while at rather high values of K_S the substrate is taken up very slow.

Next to the well known Monod equation other models have been proposed, *i.e.* the Tessier, the Moser, the Contois, and the logistic model as described in equations 5 to 8.

Tessier $$\mu = \mu_{max} \cdot \left(1 - e^{\left(-\frac{S}{K_S}\right)}\right) \qquad \text{(Eq. 5)}$$

Moser $$\mu = \mu_{max} \frac{S^n}{K_S + S^n} \qquad \text{(Eq. 6)}$$

Contois $$\mu = \mu_{max} \frac{S}{B \cdot X + S} \qquad \text{(Eq. 7)}$$

Logistic $$\mu = \mu_{max} \cdot \left(1 - \frac{X}{X_{max}}\right) \qquad \text{(Eq. 8)}$$

The models according to Tessier and Moser describe a growth kinetic that is comparable to the one described by the Monod equation. In case of equation 6, μ is converging to μ_{max} for smaller values of S when $n > 1$. The model according to Contois uses a biomass depending term instead of K_S resulting in a reduced specific growth rate at elevated biomass concentrations. This model is particular interesting for cultivations where growth is limited by the production of inhibitory compounds. The logistic model does not take the substrate concentrations into accound but is limited to the maximum bio-

mass concentration. The advantage of this model is the reduced number of parameters. Because kinetic analysis has not been carried out in the research presented here, a more detailed elaboration of this topic is abstained from.

However, several cultivation processes are compared to each other using yield coefficients. Their definitions are shown in equations 9 – 11.

$$Y_{X/S} = \left|\frac{r_X}{r_S}\right| = -\frac{dX}{dS} = \frac{X - X_0}{S_0 - S} \qquad \text{(Eq. 9)}$$

$$Y_{P/S} = \left|\frac{r_P}{r_S}\right| = \frac{P - P_0}{S_0 - S} \qquad \text{(Eq. 10)}$$

$$S_{P/X} = \left|\frac{r_P}{r_X}\right| = \frac{P - P_0}{X - X_0} \qquad \text{(Eq. 11)}$$

The biomass specific product yield coefficient in equation 11 is also called selectivity in this thesis while r_X is the growth rate, r_S the substrate utilization rate, and r_P the product formation rate. The latter one can depend on the growth rate as well as the biomass concentration as shown in equation 12.

$$r_P = \frac{dP}{dt} = \alpha \cdot r_X + \beta \cdot X \qquad \text{(Eq. 12)}$$

Product formation can be coupled to the primary metabolism ($\beta = 0$) or depend solely on the biomass concentration ($\alpha = 0$). Next to batch operation, fed batch and continuous processes are very common. However, they will not be described here any further.

High cell density cultivation processes for *Escherichia coli* are often described in the literature (Lee, 1996; Riesenberg and Guthke, 1999). Cultivation products are recombinant proteins (Schmidt *et al.*, 1999a; Schroeckh *et al.*, 1992), especially antibodies (Horn *et al.*, 1996), or polyhydroyxbutyric acid (Wang and Lee, 1998). To achieve high cell densities several aspects have to be considered. The formation of inhibitory byproducts has to be avoided and nutrients have to be available in sufficient concentrations. To avoid the adverse effects of inhibitory byproducts, cultivation parameters can be varied or the byproducts can be removed during cultivation in a dialysis bioreactor as described by Märkl *et al.* (1993). Another option is to use fed batch techniques, to en-

sure defined growth rates by the controlled addition of a concentrated feed. Several concepts for the control of feed flow can be applied (Voß, 2001).

The first cultivation processes for pharmaceutical plasmid DNA manufacturing were based on cultivations that were used for recombinant protein production. However, different pathways are involved in product formation. Instead of an increased transscription and translation a higher replication is desired. In general, defined and semidefined media are used for this purpose that contain all important nutrients for cell growth. Regulatory authorities imply to use no animal derived components (EMEA, 2001) to minimize the risk of transmitting animal spongiform encephalopathies. Therefore, defined media should be favored for plasmid production. Additionally, the concentration of all components is known and can be determined throughout the whole cultivation process with sophisticated process analytical techniques.

The basic design of gene therapy vectors has already been outlined in a preceding section. It should be noted that regulatory authorities recommend to avoid genes coding for ampicillin resistance and the application of this antibiotic during cultivation because it can lead to allergic reactions even at low concentrations. Kanamycin is however tolerated (FDA, 1998). Completely avoiding the use of antibiotics is the most preferred solution.

Bioreactor cultivations for plasmid DNA production have been described by Reinikainen *et al.* (1989) for the first time. The influence of temperature and pH on the average plasmid copy number (in the following also entitled as product selectivity $S_{P/X}$) during cultivations on semidefined media was analyzed. For sufficient segregational plasmid stability ampicillin was added to the cultivation medium. They could show that plasmid replication was more efficient under less optimal growth conditions at 30 °C and pH 6.2 – 6.8. In these early studies no statement was made with respect to plasmid form distribution.

The cultivations of a pUC plasmid carrying the temperature sensitive point mutation in the RNA II gene was described by Lahijani *et al.* (1996). A change of the cultivation temperature during exponential growth to 42 °C led to a significant increase in plasmid copy number. Batch cultivations on a semidefined medium showed plasmid concentrations of 37 mg L^{-1} while concentrations of 219 mg L^{-1} were achieved with fed batch

operation. However, the produced plasmid DNA was very inhomogeneous with larger amounts of multimeric DNA. Chen *et al.* (1997) described a pH and dissolved oxygen controlled fed batch operation for the production of plasmid DNA. The biomass concentration was high with 105 g L^{-1} and plasmid concentration reached a level of 98 mg L^{-1} giving a comparably low product selectivity of 0.9 mg g^{-1}. Schmidt showed that antibiotic free production of plasmid DNA could be accomplished by dissolved oxygen controlled fed batch cultivations on semidefined glycerol yeast media and defined glycerol media (Schmidt, 1998; Schmidt *et al.*, 1999c). Plasmid DNA concentration reached values of 222 mg L^{-1} and the corresponding biomass concentration was 57 g L^{-1} ($S_{P/X}$ = 3.9 mg g^{-1}) in case of the semidefined medium and P = 100 mg L^{-1}, X = 48 g L^{-1}, and $S_{P/X}$ = 2.1 mg g^{-1} when using the defined medium. In both cases the product was highly homogeneous.

Voss *et al.* showed that high cell density cultivation for plasmid production using a defined glycerol medium supplemented with ammonium chloride and glutamic acid could be achieved in simple batch operation (Voss *et al.*, 2004).

Carnes *et al.* (2006) developed a cultivation process using a semidefined medium based on glycerol. Exponential feeding allowed controlled growth at low specific growth rates. With pBR322 based plasmids product concentrations of 260 mg L^{-1} were achieved while pUC based plasmids reached a concentration of 1.1 g L^{-1}. The increased concentration of the pUC plasmid was accomplished by utilizing the temperature sensitive point mutation in the RNA II gene.

It should be noted, that most of the cultivations described for plasmid DNA production work with semidefined media. The use of defined ones has only been described in two instances (Schmidt, 1998; Voss *et al.*, 2004).

The biomass produced is separated from the culture supernatant by centrifugation or cross flow microfiltration and subjected to subsequent downstream processing that is described in the following sections.

3.6 Downstream Processing of Plasmid DNA

Several unit operations well known from chemical engineering can be utilized for the purification of bioproducts. As first step, intracellular products have to be isolated from the host cell by suitable cell disruption processes. Subsequently, cellular debris has to be removed by solid liquid separation and the product carrying liquid can be processed further. In case of extracellular products, the medium is separated from the producing cells and applied to downstream processing operations.

For cell disruption several processes can be applied depending on the localization of the product, its chemical and physical stability, as well as the process scale (Middelberg, 1995). These processes can be classified into mechanical and non mechanical ones while the latter one can be subclassified into physical, chemical, and biological operations. Mechanical disruption processes can use biomass in suspension or as a solid. High pressure homogenization and bead milling are commonly applied because they can either be scaled up very easily or can be used in a continuous mode of operation. Physical lysis procedures comprise osmotic shock and thermolysis while chemical lysis is achieved with chelating agents, chaotropes, detergents, solvents, and acids or bases. Biological procedures are considered to be the application of lytic enzymes, autolysis, and the utilization of molecular biology tools for product release, *i.e.* secretion of proteins by leader peptides in combination with bacteriocin release proteins. The latter one is stricktly not a lytical procedure because the cells stay intact during product release (Kleist *et al.*, 2003; Miksch *et al.*, 2002).

Centrifugation and filtration (preferably in the form of membrane separations using cross flow systems or hollow fibre modules) are used as solid liquid separations for the removal of cellular debris after lysis or for the separation of the producing cells from the cultivation medium. Subsequently, the product has to be captured and is in this way concentrated and separated from major impurities. Liquid-liquid extraction is a frequently used unit operation in chemical engineering which has also found applications in the purification of biotechnology products, *i.e.* the extraction of antibiotics like penicillin G or the extraction of proteins by aqueous two-phase systems (ATPS) and reverse micellar two-phase systems. The latter ones are also applied for the purification of plasmid DNA and are therefore discussed in more details later. In addition, extraction

processes can directly be applied to unclarified product streams making a preceding clarification unnecessary.

Chromatography is a widely used separation technique in bioprocessing. For protein separation, several different action principles can be applied, *i.e.* ion exchange chromatography, reverse phase and hydrophobic interaction chromatography, and affinity chromatography. Next to these action principles, different carrier systems are available that also have an influence on the chromatographic separation process because of different particle diameters, porosities, and chemical compositions. In size exclusion chromatography no interaction occurs between the solute and the stationary phase and separation is only achieved by the different accessability of pore volumes inside the particles.

All unit operations described above have been described for plasmid purification as well and will therefore be discussed further in the following section.

For clinical trials as well as future therapeutics, high quality plasmid DNA has to be produced at the multi- and kilogram scale. Therefore, scalable and sustainable production processes have to be developed. The main challenges for bioprocess engineering are the separation from structurally related impurities. In addition, the desired product quality as well as process development has to be documented by sophisticated analytical techniques.

3.6.1 Quality Requirements and Regulatory Guidelines

The first plasmid purification processes were based on a simple scale up of the methods used for the isolation of cloning vectors (Schorr *et al.*, 1995). RNA removal was achieved by digestion with RNase A. The resulting ribonucleotide fragments could easily be removed by subsequent anion exchange chromatography that was used for further purification of the plasmid DNA. However, an increased amount of plasmid DNA per dose resulted in higher quality demands. Commonly accepted quality parameters and corresponding analytical techniques are summarized in Table 1.

Table 1 Generally accepted quality parameters for pharmaceutical grade plasmid DNA (Voß, 2008).

Criteria	Specification	Analytical technique
Endotoxins	< 10 E.U./mg pDNA	LAL assay
Chr. DNA	< 50 µg/mg pDNA	Realtime PCR
RNA	n.d.	Agarose gel electrophoresis
Protein	< 3 µg/mg pDNA	BCA assay
Ccc content	> 95 %	CGE
Identity	Size	Restriction digest and AGE

The specifications given here usually represent the detection limit of the analytical method for a concentrated plasmid solution (> 1 g L^{-1}) as the final product. Some of the methods are not completely applicable as analytical techniques in process development or too vague as in the case of RNA detection.

To meet the high quality demands and to overcome problems with scale up of the early and simple techniques, more sophisticated production processes were developed. Furthermore, regulatory authorities demand to omit any animal derived substances throughout the whole manufacturing process (EMEA, 2001). This rules out the application of RNase A from bovine origin which has to be compensated by other methods.

Several process option can be considered for this purpose and are presented in Figure 10.

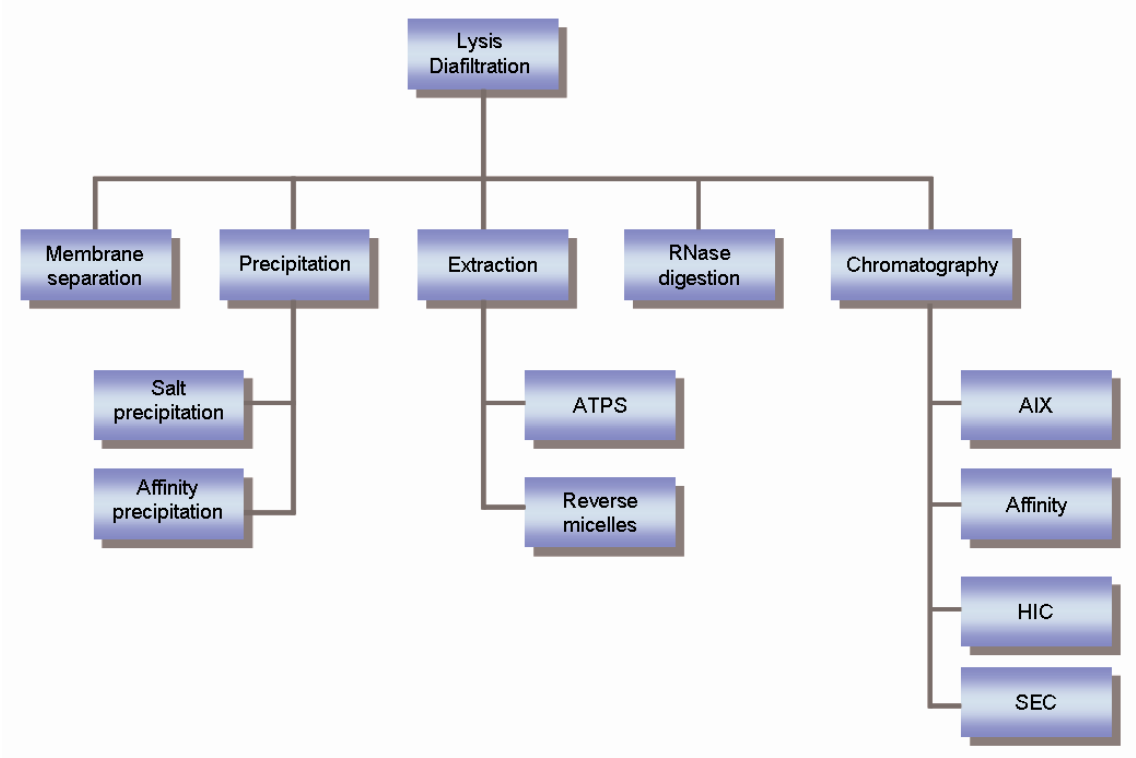


Figure 10 Process options for initial purification (AIX: anion exchange chromatography; HIC: hydrophobic interaction chromatography; SEC: size exclusion chromatography).

3.6.2 Cell Disruption

Cell disruption and the liberation of plasmid DNA is the first step in every plasmid purification protocol. Mechanical procedures have been described by Carlson *et al.* (1995). It was found that the plasmids were degraded by shear forces and product recovery was significantly low. The contamination of the product stream with fragments of chromosomal DNA was also observed but not quantified.

Alkaline lysis that has been described by Birnboim and Doly (1979) was better suited for plasmid isolation and was therefore used frequently. It is not only a lysis procedure but also a prepurification step. Under alkaline conditions the DNA double helix dissociates. Subsequent neutralization leads to rehybridization of the double strands. In case of plasmid DNA no mismatches occur due to the constraints of the circular structure. Chromosomal DNA has mismatched hybridization leading to the precipitation of aggregates. Furthermore, proteins are precipitated as potassium dodecylsulfate complexes along with the chromosomal DNA and other cell debris. The viscoelastic precipitate can

be removed by centrifugation and subsequent filtration resulting in a cleared lysate. The time course of this procedure and optimization of the reaction conditions has already been described (Ciccolini *et al.*, 1998; Clemson and Kelly, 2003).

Problems arising with scale up of this procedure are found in efficient mixing of the liquids. Insufficient mixing will result in extreme pH values which will either degrade the plasmid DNA (pH > 12.5) or result in an incomplete lysis at low pH. Too vigorous mixing degrades the precipitated chromosomal DNA and smaller fragments are solubilized again, thus contaminating the product stream (Levy *et al.*, 2000). The large size distribution of these fragments makes analytical detection as well as removal difficult. Several concepts have been developed to minimize these shear forces and ensure efficient mixing. Continuous mixing of the liquids is a common principle in these processes. In several cases, mixing is achieved by static (Urthaler *et al.*, 2007; Wan *et al.*, 1998) or active mixers (Hebel *et al.*, 2006). Simple T-connectors have also proven to be very effective when using sufficient flow rates (Voss, 2007; Voss *et al.*, 2005) which will be outlined in more details in the results section.

The removal of the precipitated debris is still achieved by centrifugation and additional filtration which is a rather tedious and time consuming procedure at the larger scale. Cross flow filtration or depth filtration cannot be applied in this case because the gel like precipitate quickly blocks the pores of the filter material.

As an alternative to alkaline lysis thermal disruption of *E. coli* has been described for plasmid isolation (Holmes and Quigley, 1981). Biomass can be harvested by filtration using a filter aid and the cells are disrupted by rinsing with a hot buffer (O'Mahony *et al.*, 2005). Part of the contaminating RNA and chromosomal DNA could already be removed at this stage by a proper choice of temperature and buffer composition.

The cleared lysate obtained after alkaline lysis has a composition as described in Table 2. Although proteins constitute the majority of impurities, structurally related compounds like chromosomal DNA, lipopolysaccharides, and RNA are the most difficult ones to separate from plasmid DNA.

Table 2 Composition of a bacterial cleared lysate (Stadler *et al.*, 2004).

Compound	Relative amount / %
Proteins	55
Chr. DNA	3
LPS	3
Plasmid DNA	3
RNA	21
Others	15

RNA is the largest fraction of nucleic acids with 21 % while plasmid DNA constitutes only 3 %. Therefore, suitable processes have to be developed for the separation of these two molecular species. In addition, these methods should be capable of depleting other impurities as well. To simplify subsequent purification, this separation step would capture the plasmid DNA while RNA is removed. The following purification steps will remove the other impurities until the desired product quality is obtained.

3.6.3 Recombinant RNase

Because the application of RNase A from bovine origin is ruled out by regulatory guidelines (EMEA, 2001), it might be substituted by a recombinant RNase produced in *E. coli*. The recombinant expression of the eukaryotic RNase A is certainly afflicted with the same difficulties that are encountered for other eukaryotic proteins as well, *i.e.* different codon usage, incorrect folding, *etc.*. An attractive alternative was found in the well characterized RNase from *Bacillus amyloliquefaciens* (RNase Ba, Barnase). This enzyme is a 12 kDa protein lacking disulfide bonds (Hartley and Rogerson, 1972). Its three dimensional structure is shown in Figure 11. The ribonucleolytic activity of Barnase is suppressed by coexpression of the 10 kDa inhibitor called barstar (Hartley, 1989). Figure 12 shows the interaction of both proteins. In *B. amyloliquefaciens* RNase Ba is secreted in the late exponential and stationary phase to hydrolyze RNA molecules in the surrounding medium and use the products as substrates (Hahnen *et al.*, 2000). The enzyme has a pI of 8.4 and is considerably stable at elevated temperatures (T_m = 50 °C)

and at a broad pH range (pH 3 – 10). Its catalytic optimum is found at pH = 8.5 (Hartley, 1989; Mossakowska *et al.*, 1989). The recombinant expression of Barnase in *E. coli* proved to be a challenge for molecular biologists because the regulatory elements for gene expression had to be chosen very properly to guarantee non lethal expression of the RNase.

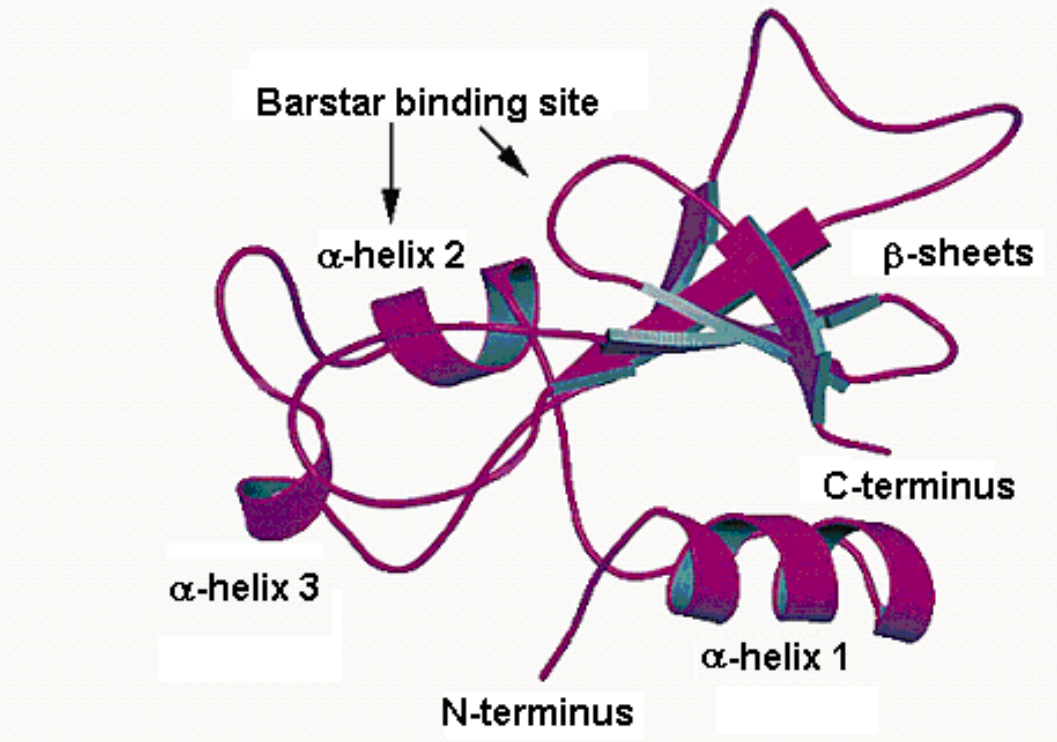


Figure 11 Three dimensional structure of RNase Ba (Huang *et al.*, 1999).

By now, several expression systems are available that differ in the promoter sequences and fusion elements. The inhibition of RNase Ba by Barstar is accomplished by the formation of a non covalent complex blocking the active centre of the enzyme. Figure 12 shows a schematic of this complex formation and the amino acids taking part in the interaction. Other bacterial RNases having a comparable structure to Barnase (*i.e.* RNase Sa, Sa2, Sa3, St from Streptomyces) are also inhibited by Barstar (D'Alessio *et al.*, 1997). Homologous proteins to Barstar could be found in other microorganisms as well (D'Alessio *et al.*, 1997). The Barnase-Barstar complex shows a dissociation constant of $K_D = 10^{-14}$ M. The highest affinity between Barnase and its inhibitor is found at a pH range of 7.0 – 9.0 and at pH 4.5 or lower the interaction is completely suppressed.

Ribonucleases are phosphodiesterase enzymes that saponificate the RNA substrate between the 5'-phosphate group and the 3'-OH group of a neighboring nucleotide. Barnase and RNase A differ in their structure and in their base preference. However, both enzymes follow the same catalytic mechanism (Mossakowska *et al.*, 1989). RNase Ba cleaves RNA preferentially at the 3'-end of guanosine residues.

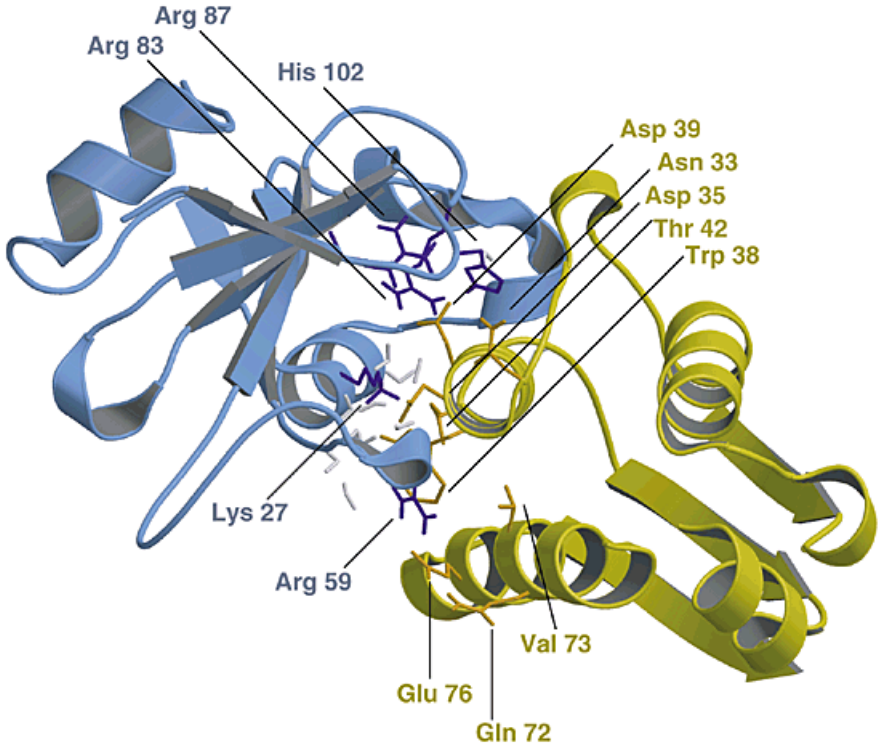


Figure 12 Schematic presentation of the interaction of barnase (blue) with its cytosolic inhibitor barstar (yellow). Amino acids taking part in the interaction are shown in dark blue (Lee and Tidor, 2001).

Additionally, a preference for adenosine residues as the second binding site is observed with the following order: GpA > GpG > GpC ~ GpU (Mossakowska *et al.*, 1989).

The recombinant RNase can be produced by periplasmic translocation and subsequent release from the periplasm after acidification with acetic acid (pH 4 – 4.5) as shown by Okorokov *et al.* (1994). A few methods have been described for Barnase purification. One process captures the enzyme by batch adsorption on a cation exchange resin (SP Trisacryl) and subsequent step elution with 2.0 M ammonium acetate at pH 8.0 (Okorokov *et al.*, 1994). Further purification is achieved by reversed phase chromatography using a Silasorb C18 SPH column. Other methods use immobilized metal chelate

affinity chromatography (IMAC) for the purification of a His tagged version of the protein (D'Alessio *et al.*, 1997) or the interaction with Barstar as an affinity system (Ramachandran and Udgaonkar, 1996).

In this thesis, the recombinant expression and purification of Barnase and its application in a plasmid purification process is described.

3.6.4 Extraction Processes

Liquid-liquid extraction is a well known and frequently used unit operation in chemical engineering. It can be scaled up easily and works with inexpensive chemicals and equipment. Extraction processes are described by several parameters. The selectivity α (equation 13) represents the efficiency of a system to separate two components A and B from each other. In equation 13, x_A and x_B represent the molar fractions of the components in the two phases respectively.

$$\alpha_{AB} = \frac{x_{A'} / x_{B'}}{x_{A''} / x_{B''}} \qquad \text{(Eq. 13)}$$

The partitioning of a component is described by the Nernst equation (equation 14) where K is the partition coefficient, and c_E and c_R the solute concentrations in the extract and in the raffinate phase at equilibrium conditions.

$$K = \frac{c_E}{c_R} \qquad \text{(Eq. 14)}$$

The degree of extraction ϕ_E is given in equation 15.

$$\phi_E = \frac{V_E}{V_R} \cdot \frac{c_E}{c_R} = F \cdot K \qquad \text{(Eq. 15)}$$

The extract phase volume is represented by V_E, while V_R is the raffinate phase volume, and F is the phase volume ratio. The degree of extraction constitutes the relative amount of the solute in both phases.

In biochemical engineering extraction processes using organic-aqueous systems are state of the art for the purification of antibiotics. Proteins and nucleic acids cannot be

extracted by apolar solvents because of the multiionic character of these biopolymers and they might even be denaturated in the presence of these solvents. Therefore, other systems have been developed that allow partitioning between two aqueous phases. In case of aqueous two-phase systems (ATPS) two immiscible polymer solutions or a polymer and a salt solution are applied. These systems are well known for decades and have been decribed for protein purification on several occassions as outlined by the following examples (Azevedo *et al.*, 2007; Duarte *et al.*, 2007; Fexby *et al.*, 2004; Gu and Glatz, 2007; Johansson *et al.*, 2008; Kula, 1990; Li and Beitle, 2002; Poppenborg *et al.*, 1997; Selber *et al.*, 2000; Thömmes *et al.*, 2001). Furthermore, the extraction processes have been optimized by the introduction of thermoseparating polymers (Galaev and Mattiasson, 1993) making a back extraction from the polymer phase unnecessary. More detailed information is given in comprehensive overviews by Albertsson (1986) and Kula (1990).

The second system that is described for the partitioning between two aqueous phases is reverse micellar extraction. Here, an aqueous phase is encapsulated in reverse micelles that are formed by charged surfactants in an apolar organic solvent (Hatton, 1989). When contacting these phases with an aqueous feed phase the proteins can be partitioned in between. Separation can take place during forward and back extraction. The pH and the ionic strength in the aqueous phase are considered to be the main influencing parameters for the partitioning because they have an effect on the electrostatic interaction between the charged headgroup of the surfactant and the charged biopolymer. Additionally, the distribution is also affected by the size of the reverse micelles that changes with the ionic strength in the system (Göklen and Hatton, 1987). The most common surfactants used for these processes are sodium bisethylhexyl sulfosuccinate (AOT) and methyltrioctylammonium chloride (TOMAC). Additionally, several other parameters have been investigated, *i.e.* the influence of salt type (Leser *et al.*, 1986) and the effect of the co-surfactant (Hong and Kuboi, 1999).

The capacity of the reverse micellar phase is determined by the surfactant concentration (Hentsch *et al.*, 1992). At low concentrations partitioning is limited by the number of reverse micelles. For α-chymotrypsin in AOT-isooctane systems values around 10 mM AOT were found to be optimal. An effect on the kinetic of extraction could not be observed in this case. The specifity of the reverse micellar extraction could be increased

by the introduction of affinity surfactants. Bioaffinity (Woll *et al.*, 1989) as well as immobilized metal chelate affinity (Poppenborg and Flaschel, 1994; Poppenborg *et al.*, 1997) have been analyzed. In both cases the separation efficiency could be increased significantly.

The distribution of nucleic acids has been analyzed in both systems. In case of aqueous two-phase systems basic research was also conducted by Albertsson (Albertsson, 1965) testing the influence of electrolytes, polymer concentration, and nucleic acid conformation in dextran-polyethylene glycol (PEG) systems. However, these fundamental investigations did not get significant attention in the case of nucleic acids. This changed with the application of plasmid DNA in genetic medicine. During the search for robust and scalable purification protocols the early research was picked up again. Additional publications describing the partitioning of plasmid DNA in PEG-dextran aqueous two-phase systems (Ohlsson *et al.*, 1978) led to the development of several extraction procedures. Ribeiro *et al.* (2002) described the purification of plasmid DNA from bacterial cleared lysates by systems based on polyethylene glycol and potassium phosphate. By using PEGs of different molecular masses between 200 and 8000 g mol^{-1}, they were able to identify systems that were capable to deplete some impurities. With respect to plasmid DNA purity, a PEG-600 system with 40 % lysate load proved to be very efficient. However, product recovery was only moderate with 34 and 44 % because a large amount of the plasmid precipitated at the interphase due to the high PEG and salt concentrations. Other systems described by the authors showed better product recoveries, but on the other hand purification was less optimal. In general, no significant concentration could be achieved with all the systems described. Subsequent purification for further depletion of contaminants could be achieved by the same group (Trindade *et al.*, 2005). By using an enhanced PEG-600-$(NH_4)_2SO_4$ extraction system they were able to recover the plasmid DNA in the salt phase that was further purified by hydrophobic interaction chromatography. In comparison to a purification scheme based on sequential precipitation with isopropanol and ammoniumsulfate followed by HIC, they were able to prepare plasmid DNA in comparably high purity. However, the extraction process showed only a product recovery of 75 % in comparison to the precipitation based process having a recovery of 87 %. Additionally, the systems did not meet the high quality demands that were outlined in Table 1.

Systematic screening could identify more optimal extraction systems for plasmid DNA (Frerix *et al.*, 2005). When using PEG-800-citrate systems plasmid recovery was 85 %, while 80 % of the contaminating RNA has been removed. With PEG-600-phosphate systems a recovery of 60 – 75 % was observed with 89 % RNA removal. The authors were also able to integrate the extraction process into scalable purification schemes (Frerix *et al.*, 2007) based on membrane filtration and/or ion exchange chromatography.

Other systems utilized thermoseparating polymers for initial purification (Kepka *et al.*, 2004b). Here, plasmid DNA was partitioned between two phases formed by the addition of an ethyleneoxid-propyleneoxid-copolymer ($EO_{50}PO_{50}$) and dextran T-500. Plasmid DNA is efficiently transferred to the copolymer phase while 80 % of the contaminating RNA and 36 % of the total protein remained in the dextran phase. Thermoprecipitation of the copolymer at 55 °C left the plasmid DNA along with some RNA (20 %) in a pure aqueous phase, thus making further purification more simple. But since the product was still contaminated with 20 % of the RNA an additional purification step was absolutely necessary. A solution was presented by the same authors (Kepka *et al.*, 2004a) using the so called lid bead particles (Gustavsson *et al.*, 2004). This chromatographic material is a restricted access matrix with a non charged outer surface and a positively charged pore surface that works as an anion exchanger and is capable to selectively bind RNA and proteins while the plasmid DNA cannot penetrate the pores and is excluded.

The dissolution of nucleic acids in reverse micellar phases has only been described in a few instances. Imre and Luisi (1982) injected small amounts of an aqueous DNA solution into a reverse micellar phase consisting of AOT in isooctane. The DNA was completely solubilized and retained its double helical structure. The CD spectra of the DNA suggested a condensed ψ-form. These results have been confirmed by Pietrini and Luisi (2002) more recently. They also analyzed the size distribution in the population of reverse micelles by light scattering studies and found three different species with the size of 5 nm, 100 nm, and 1000 nm. While the 100 nm aggregates contained DNA in a normal spectral state and in a condensed form the 1 μm aggregates contained only uncondensed DNA, but were comparably unstable. It was assumed that these larger structures constitute clusters of smaller reverse micelles. Therefore, this work was considered as one of the key publications and in conclusion it was assumed that selective separation of plasmid DNA from RNA by reverse micellar solutions might be possible. The extrac-

tion of short, linear DNA fragments from salmon testes with reverse micellar phases consisting of a cationic surfactants in isooctane from aqueous solutions has been described by Goto *et al.* (1999) for the first time. The partitioning was analyzed in these systems in the presence of aqueous salt solutions. During further research (Goto *et al.*, 2004) the influence of cosurfactants on the partitioning behaviour of the nucleic acid fragments was analyzed for forward and back extraction. It was concluded that the electrostatic interaction between the cationic surfactant and the negatively charged DNA backbone is the driving force for forward extraction while the back transfer of the DNA is accomplished by the proper choice of the alcohol. Based on these results, the capability of reverse micellar systems comprising cationic surfactants in an apolar solvent was analyzed and used to develop an extraction process for pharamaceutical plasmid DNA purification.

3.6.5 Affinity Purification

Affinity separation is the most selective purification method. For proteins immobilized metal chelate affinity procedures (Porath, 1992) as well as protein A chromatography especially for antibody purification (Hahn *et al.*, 2005) are well known techniques. Several other affinity systems are applied in protein purification, but in the case of nucleic acid separation only a few systems have been described yet. The formation of a triple helix by an immobilized oligonucleotide and a corresponding double stranded DNA was used for selective purification of DNA by Ito *et al.* (1992). Schluep and Cooney (1998) as well as Costioli *et al.* (2003) used this interaction for plasmid purification. In the first case, a 15mer oligonucleotide was immobilized to a large pore chromatographic support and used to isolate a pUC19 derivative containing the recognition sequence for triple helix formation. Product yield was 68 % with pure plasmid DNA as a model system and 37 % when using a bacterial cleared lysate as starting material. Although, this method was capable to completely remove RNA, no chromomsomal DNA was detectable by HPLC analysis and most of the protein was depleted, the method did not find a broad application. The reason for this is found in the low chemical and biochemical stability of the immobilized oligonucleotide and the very slow kinetic of triple helix formation as well as the low product recovery. Nevertheless, the same affinity interaction was exploited by Costioli *et al.* (2003) in a precipitation process. They immobilized a short

oligonucleotide sequence $(CTT)_7$ to a thermoresponsive N-isopropylacrylamide oligomer forming an affinity macroligand (AML). At low temperatures (4 °C) the AML stayed in solution and bound specifically to the target DNA and the resulting complex could be precipitated by raising the temperature above the critical solution temperature of the thermoresponsive oligomer to 40 °C. Redissolution at lower temperatures and a subsequent pH shift to pH 9.0 released the plasmid DNA from the complex resulting in a highly pure product. The authors were able to recover the plasmid DNA from a bacterial lysate with 62 % yield which is considerably high for triple helix affinity but on the other hand only a moderate value with respect to other purification methods. The specific protein concentration of 0.5 mg mg^{-1} in the purified DNA was also far above the generally accepted value of 3 μg mg^{-1} (Table 1). Nevertheless, the authors were able to separate the plasmid DNA from the contaminating RNA and plasmid DNA was concentrated very effectively.

Protein-DNA interaction is a common phenomenon observed in nature. Several enzymes as well as regulatory proteins contain DNA binding motives and bind to specific sequences. However, this affinity system has only been described occasionally for plasmid purification. Lundeberg *et al.* (1990) used an immobilized lac repressor protein for selective binding of DNA fragments containing the lac operator sequence. This system was also applied by Kumar *et al.* (1999) and analyzed for its capability of sequence specific binding of DNA. Batch adsorption experiments with LacI sepharose showed that 40 % of genomic DNA lacking the operator site were adsorbed on the affinity resin while a slightly higher binding ratio of 60 % was observed when using a linearized plasmid DNA containing the recognition sequence. Although binding of nucleic acid was observed no separation from other biomolecules was shown in this report. Nevertheless, the principle of protein-DNA interaction as a potential tool for the isolation of nucleic acids was introduced here. Woodgate *et al.* (Woodgate *et al.*, 2002) used an immobilized zinc-finger protein for chromatographic affinity capturing of plasmid DNA. The system was further analyzed with respect to binding capacities on different stationary phases by Ghose *et al.* (2004). Further research from this group was done with an immobilized peptide containing the DNA binding domain of LacI (Forde *et al.*, 2006). Here, a focus was also set on the dynamic binding capacity and no data regarding selectivity was presented.

Immobilized metal chelate affinity chromatography (IMAC) is a well known affinity technique used for the purification of recombinant proteins. Purine bases also show an heterocyclic nitrogen that could probably form metal complexes as shown in Figure 13. In intact double stranded DNA this nitrogen is blocked because opposite bases hybridize via hydrogen bonds.

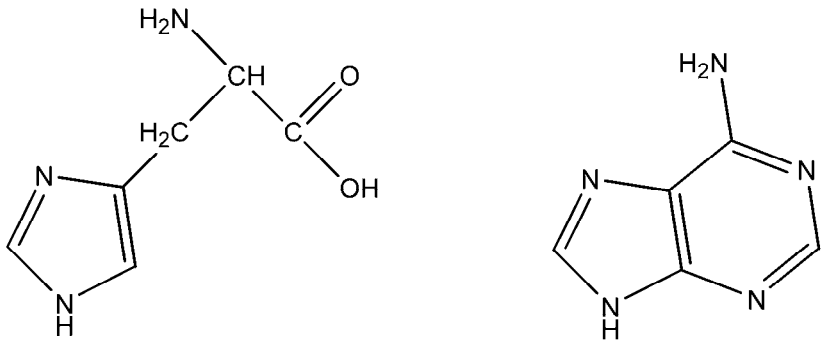


Figure 13 Comparison of histidine and adenine.

On the contrary, RNA as well as degraded DNA show more or less extended single stranded areas, both exposing purine bases that can form metal complexes. The application of this principle in plasmid purification has been shown by Balan *et al.* (2003) for the first time. They used a copper charged copolymer of N-isopropylacrylamide (NIPAM) and vinyl imidazole (VI) to selectively precipitate the RNA from a bacterial cleared lysate in the presence of 800 mM NaCl. This interaction was analyzed more thorougly with respect to different coordination centres and a negative chromatography process has been presented for plasmid purification (Murphy *et al.*, 2003). Cano *et al.* (2005) could show that even genomic DNA could be separated from plasmid DNA utilizing this technique in combination with selective renaturation of the plasmids.

The chromatographic procedure has been optimized further with respect to more efficient binding of RNA to the matrix by the addition of solutes such as ethanol, methanol, 2-propanol, 1-propanol, and dimethyl sulfoxide (Potty *et al.*, 2006) and with respect to the elution conditions (Fu *et al.*, 2006).

3.6.6 Chromatographic Matrices for Plasmid Purification

Chromatography is a commonly used separation technique in biotechnological processes because of its high selectivity in comparison to other methods.

Several chromatographic procedures have been described for plasmid purification. Anion exchange chromatography is used in most cases because the negatively charged backbone of the nucleic acid can interact with the positively charged ion exchange groups (Ferreira *et al.*, 1998; Schorr *et al.*, 1995). Anion exchange chromatography has been used in different forms. Ferreira *et al.* worked with porous beads that have been designed for the purification of proteins in a normal chromatographic mode (1998) or in the form of expanded batch adsorption (2000). Hydrophobic interaction chromatography (HIC) has also been applied for plasmid purification (Diogo *et al.*, 2001a; Diogo *et al.*, 2001b). Interesting results have been observed by Lemmens *et al.* with thioaromatic groups for HIC purification of plasmids (2003). In the presence of 2 M ammonium sulfate this material (Plasmid Select, GE Healthcare) selectively bound the supercoiled form of the plasmid DNA while the oc form was not retained. The HIC step was preceded by a size exclusion chromatography (SEC) carried out to separate the plasmid DNA from RNA in a group separation mode. Final polishing was achieved after HIC with an anion exchange chromatography on Source-30 Q that depleted several impurities and especially endotoxins. In this way, highly pure supercoiled plasmid DNA was obtained that met the specifications outlined in Table 1. Although the process is comprised of three chromatographic steps that are strongly interconnected with each other, its main bottleneck is the size exclusion chromatography in the beginning. This operation is rather tedious and time consuming and does not result in product concentration but in a slight dilution of the sample. As a consequence, the subsequent HIC step also suffers from long sample loading time. Although the cleared lysate can be concentrated by ultrafiltration prior to SEC, this option is only discussed in theory and is certainly afflicted with additional product loss and processing time. Furthermore, extra equipment is necessary for this step increasing overall process costs. The size exclusion step was optimized by the development of the so called lid beads (Gustavsson *et al.*, 2004). As already mentioned, this matrix is a restricted access material that has anion exchange groups in the pores and an inert hydrophilic outer shell. RNA can enter the pores and is

sufficiently retained by interaction with the anion exchanger while plasmid DNA remains in the void volume and is not retained at all. This method has been applied for the subsequent purification of extracts from aqueous two-phase systems that has already been outlined before (Kepka *et al.*, 2004a).

The application of porous beads for plasmid DNA purification is afflicted with serious limitations. Originally these matrices have been designed for protein purification and offer a high surface area for binding mainly inside the pores. Plasmid DNA is considerably larger than proteins with sizes in the sub μm range and molecular masses over 10^6 g mol^{-1} while proteins have a globular structure with 2 – 10 nm in diameter (He and Niemeyer, 2003; Tyn and Gusek, 1990). Typical diffusion coefficients for plasmid DNA are in the order of 10^{-8} cm^2 s^{-1} (Fishman and Patterson, 1996) while that of proteins or virus particles of an equivalent mass are one order of magnitude larger (He and Niemeyer, 2003; Tyn and Gusek, 1990). The mass transport into and inside porous beads is accomplished by diffusion processes which are very slow for plasmid DNA. In addition, dynamic capacities at common flow rates are suffering from this slow diffusion. Furthermore, plasmid DNA might not at all be able to penetrate the pores and thus utilize the binding sites inside of the beads. This fact was demonstrated by Ljunglöf *et al.* (1999) using confocal scanning laser microscopy. Due to these facts, anion exchangers or HIC matrices based on porous beads show a low dynamic capacity of about 1 mg mL^{-1}. Higher values are obtained for materials having a branched ligand structure, *e.g.* Fractogel, that shows capacities about 3 mg mL^{-1} (Eon-Duval and Burke, 2004; Urthaler *et al.*, 2005).

Supermacroporous materials like monolithic columns have also proven to be well suited for plasmid purification. These matrices are a single particle block or disk that is pervaded by an interconnected pore system. The sample is passing through these pores by convective flow resulting in flow independent dynamic capacities (Strancar *et al.*, 2002). Due to an optimized pore diameter high capacities have been described for plasmid DNA with values up to 8 mg mL^{-1} (Urthaler *et al.*, 2005). By further increasing the pore size, these materials could even be penetrated by larger particles like viruses (Kramberger *et al.*, 2004) or even whole cells (Arvidsson *et al.*, 2002; Dainiak *et al.*, 2005), but the capacity decreased in these instances. The chemistry for the synthesis of these monoliths is relatively simple and only a few polymers are utilized for this pur-

pose (Svec and Huber, 2006; Xie *et al.*, 2002). In case of CIM stationary phases (Strancar *et al.*, 2002) a copolymerization of glycidyl methacrylate and ethylene dimethacrylate in the presence of porogenic solvents results in the formation of a rigid polymer with epoxy groups that could be further functionalized (Viklund *et al.*, 1997). Pore size is determined by the composition and the chemical properties of the porogenic solvents as well as the reaction temperature. A serious problem that is encountered for methacrylate based monoliths is the difficulty of preparing larger columns because the polymerization reaction is exothermic. Due to insufficient heat dissipation in larger reaction volumes, columns with large variations in the pore size distribution having unpredictable flow characteristics were obtained (Strancar *et al.*, 2002). However, this problem could be circumvented by setting up larger columns out of several annuli that fit into each other (Podgornik *et al.*, 2000).

Another material that has recently gained attention in the literature are the so called cryomonoliths. For the preparation of these stationary phases acrylamide and ethylene bisacrylamide were used as monomers. The polymerization is the same as the one used for the preparation of polyacrylamide gels for electrophoretic protein analysis. However, in the case of cryogels the polymerization is carried out at low temperatures (usually less than -10 °C). Under these conditions the water in the reaction mixtures freezes and acts as the porogenic substance while the reaction is taking place in the remaining liquid phase. Because of the relatively high concentrations of monomers in the liquid phase the reaction also proceeded at a reasonable rate at low temperatures. However, pore size was found to be depending on the reaction temperature and the speed at which the mixture was frozen. These parameters were difficult to control and in this way materials with reproducable properties were difficult to achieve. The cryogels have also been used for plasmid purification (Hanora *et al.*, 2006). Because the basic highly porous matrix showed a very low binding capacity the amount of ligands was increased by graft polymerization.

4 Results and Discussion

4.1 Cultivation Strategies

4.1.1 Cultivations on Defined and Semidefined Media

Semidefined media like HSG medium allowed the production of high quality plasmid DNA at moderate concentrations. HSG is based on glycerol as the carbon source. In addition, it contained soybean peptone and yeast extract as described by Voß *et al.* (2003). A complete list of media compositions is given in the appendix section. Common cultivation parameters achieved during batch operation were X = 7 – 8 g L^{-1}, P = 25 mg L^{-1}, $S_{P/X}$ = 3.5 mg g^{-1}. During cultivation a good segregational plasmid stability was observed and no significant loss of product during an extended stationary cultivation phase was noted. Plasmid quality with respect to form distribution was acceptable over the whole production process. By using fed batch techniques it was possible to develop a cultivation process for high cell density plasmid production (Schmidt 1998). However, the most serious disadvantage of this medium was found in its composition since it contained large amounts of soybean peptone and yeast extract. These components are not ideally suited for pharmaceutical production because extracts might be prepared by using animal derived enzymes and their composition can vary. Both facts would rule out this medium for GMP production. Therefore, the use of a fully defined (synthetic) medium is recommended.

First attempts to develop a defined medium based on the semidefined HSG medium were carried out by Schmidt (1998). He used a fed batch technique to feed glycerol and $MgSO_4$ which resulted in a process that gave product concentrations that were significantly lower in comparison to a fed batch process with a semidefined medium (defined: P = 100 mg L^{-1}, X = 48 g L^{-1}, $S_{P/X}$ = 2.1 mg g^{-1}; semidefined: P = 220 mg L^{-1}, X = 57 g L^{-1}, $S_{P/X}$ = 3.9 mg g^{-1}). In this case ammonia used for the correction of pH during cultivation constituted the only nitrogen source in both processes. A synthetic medium for batch operations resulting in high plasmid concentrations (Voss *et al.*, 2004) (P = 50 mg L^{-1}, X = 20 g L^{-1}, $S_{P/X}$ = 2.5 mg g^{-1}) was based on glycerol, ammonium chloride, and glutamic acid. It was capable to produce high plasmid concentrations in a sim-

ple batch procedure. These values are also summarized and compared to the results of this thesis in Table 14 at the end of this chapter.

4.1.2 Supplementation of a Defined Medium with Amino Acids

The differences in the medium composition obviously had an effect on plasmid replication and therefore the final product concentration in the different cultivation processes. Additionally, further research was motivated by comparable results found in other references (O'Kennedy *et al.*, 2000) showing that the biomass and product formation was depending on the amino acid source used. Therefore, we tried to supplement a basic glycerol medium (GGM) based on HSG with different amino acids. As a basis for this supplementation the amino acid composition in HSG was analyzed (Schulze-Horsel, 2003). The concentrations are presented in Table 3. In addition, ammonium sulfate was applied as a nitrogen source and trace elements as well as thiamine were supplemented separately. In order to keep the formulation of the medium simple only few amino acids were added to the GGM medium. For comparison, cultivations with the basic GGM medium lacking amino acids were analyzed (Bär, 2005). Biomass concentration was $X = 3.2\ g\ L^{-1}$ which was comparably lower than the value obtained in HSG. The reason was that the extract concentration was not compensated by additional glycerol in the GGM medium. When looking at the yield coefficient $Y_{X/S}$ it became evident that the HSG medium was more efficient (GGM: $Y_{X/S} = 0.11$; HSG: $Y_{X/S} = 0.18$). The selectivity in GGM was $S_{P/X} = 5\ mg\ g^{-1}$ which was higher than the selectivity obtained with a defined medium during fed batch operation by Schmidt (1998). However, due to the low biomass concentration the final product concentration was also low with $P = 12\ mg\ L^{-1}$. Another issue that had to be addressed was the observed product loss during the stationary phase as shown in Figure 14.

Table 3 Concentration of amino acids (AA) in HSG and supplement concentration used in GGM (3x HSG concentration) (Bär, 2005). The concentrations of L-proline and L-cysteine could not be determined. Therefore, they have been used in the same concentration as L-valine.

HSG	Molecular mass / g mol^{-1}	c(AA) / mM	c(AA) / g L^{-1}	c(AA) / g L^{-1} 3x HSG
L-alanine	90	2.6	0.234	0.702
L-arginine	88	0.4	0.035	0.106
L-asparagine	133	1.2	0.159	0.479
L-aspartic acid	133	1.4	0.186	0.559
L-cysteine	122	n/a	n/a	0.878
L-glutamic acid	147	1.8	0.264	0.794
L-glutamine	147	1.8	0.265	0.794
glycine	75	1.8	0.135	0.405
L-histidine	156	0.4	0.062	0.187
L-isoleucine	132	1.4	0.185	0.554
L-leucine	132	2.6	0.343	1.030
L-lysine	148	2.0	0.296	0.888
L-methionine	149	0.4	0.060	0.179
L-phenylalanine	161	0.6	0.097	0.289
L-serine	105	1.5	0.158	0.473
L-proline	117	n/a	n/a	0.842
L-tyrosine	178	0.9	0.160	0.480
L-threonine	128	1.4	0.180	0.538
L-tryptophane	198	0.2	0.039	0.119
L-valine	177	2.4	0.281	0.843

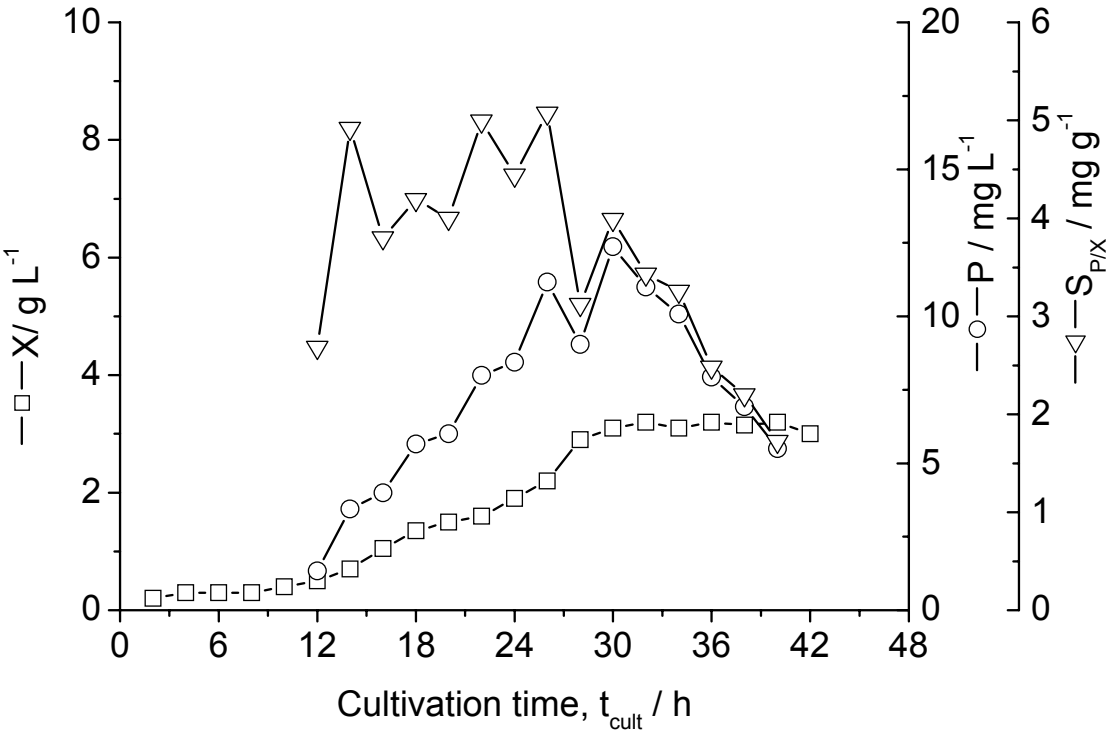


Figure 14 Batch cultivation of *E. coli* DH5α containing the plasmid pUT649 (4.6 kb) in a 2 L batch bioreactor on basic glycerol medium (GGM). The composition of GGM is given in the Appendix chapter. Oxygen saturation was kept at 60 % by increasing the stirrer frequency.

This basic medium should be supplemented with amino acids in order to obtain a medium that supported high plasmid concentrations with high amounts of the supercoiled form. A high selectivity as well as overall productivity would be most desirable. As first screening experiments the medium was supplemented with the proteinogenic amino acids according to the following schemes in Table 4 and Table 5. Compositions 1 – 4 in both schemes contained 5 amino acids while compositions 5 – 9 contained only 4. The concentration of each amino acid was 3 times the concentration determined in HSG medium.

Table 4 First scheme for the composition of GGM media supplemented with amino acids.

Scheme 1	1	2	3	4
5	arginine	alanine	asparagine	cysteine
6	isoleucine	valine	tryptophane	methionine
7	lysine	serine	glutamic acid	glutamine
8	threonine	histidine	aspartic acid	glycine
9	proline	leucine	tyrosine	phenylalanine

Table 5 Second scheme for the composition of GGM media supplemented with amino acids.

Scheme 2	1	2	3	4
5	arginine	glutamine	tryptophane	valine
6	proline	leucine	aspartic acid	histidine
7	methionine	alanine	tyrosine	glutamic acid
8	serine	phenylalanine	cysteine	asparagine
9	isoleucine	glycine	lysine	threonine

The results obtained with these media are shown in Table 6 and Table 7. According to the highest productivity obtained in these first screening experiments, amino acids from batches 4 and 5 in scheme 1 that were also present in at least one of the batches 1, 2, 6, or 9 from scheme 2 were selected for further tests, being arginine, glutamine, methionine, alanine, phenylalanine, and glycine. Additionally isoleucine and proline were also taken into further studies.

Table 6 Results obtained from cultivations according to scheme 1 in Table 4.

Scheme 1	GGM	1	2	3	4	5	6	7	8	9
X / g L^{-1}	2.5	3.7	0	1.7	3.7	3.75	3.5	2.2	4	0.6
$S_{P/X}$ / mg g^{-1}	1	3.3	0	2.3	3.8	4.6	2.5	4	1.6	0.8
L_V / mg L^{-1} h^{-1}	0.04	0.19	0	0.08	0.3	0.36	0.18	0.2	0.13	0.01

Table 7 Results obtained according to scheme 2 in Table 5.

Scheme 2	GGM	1	2	3	4	5	6	7	8	9
X / g L^{-1}	2.5	3.3	2.6	0.8	0	0	3.8	4.6	1.1	4.2
$S_{P/X}$ / mg g^{-1}	1	1.1	3	1.7	0	0	1.7	0.3	0.6	1.1
L_V / mg L^{-1} h^{-1}	0.04	0.07	0.15	0.02	0	0	0.1	0.02	0.01	0.1

Based on these 8 amino acids four different combinations were used to analyze biomass and product formation, as well as selectivity and productivity. The results obtained with the supplemented media are shown in Table 8.

Table 8 Results obtained from batchers I – IV.

Batch	I	II	III	IV
Amino acids	Arg, Pro, Gln, Met	Ile, Ala, Phe, Gly	Arg, Pro, Phe, Gly	Ile, Ala, Gln, Met
X / g L^{-1}	4.3	3.4	3.6	3.5
P / mg L^{-1}	1.5	5.9	1.2	4.9
$S_{P/X}$ / mg g^{-1}	0.3	1.8	0.4	1.5
L_V / mg L^{-1} h^{-1}	0.03	0.12	0.025	0.1

Only the batches II and IV showed comparable results to the initial screening experiments. These results indicated that the combination of amino acids had a significant influence on productivity. Taking all screening results into account the number of amino acids was reduced to six discarding proline and arginine. Based on these six amino acids 5 media containing 3 amino acids, as well as 11 media containing 2 amino acids in combination, and 6 media containing only one amino acid were prepared. Since combinations of amino acid played a vital role for productivity, the batches A to P were expected to enbable us to identify systems with increased productivity. Batches Q to V should confirm our hypothesis that single amino acids have no significant influence on production. Amino acid concentration were three times the concentration found in the semidefined HSG medium. The results are summarized in the following Tables 9 to 12.

Table 9 Results obtained with media GGM/A – GGM/E.

Batch	A	B	C	D	E
Amino acids	Ala, Gln, Gly	Ile, Met, Phe	Ala, Gln, Met	Ala, Ile, Phe	Ala, Gly, Phe
X / g L^{-1}	1.7	4.1	5.4	4.5	2.5
P / mg L^{-1}	3.3	2.5	5.4	7.2	3.2
$S_{P/X}$ / mg g^{-1}	1.8	0.6	1	1.6	1.3
L_V / mg L^{-1} h^{-1}	0.07	0.04	0.1	0.16	0.07

Table 10 Results obtained with media GGM/F – GGM/K.

Batch	F	G	H	I	J	K
Amino acids	Ala, Gln	Ala, Gly	Ala, Ile	Ala, Phe	Gln, Ile	Gln, Phe
X / g L^{-1}	1.6	2.1	4.2	1.6	4.2	3.5
P / mg L^{-1}	4.3	2.7	12.2	3.2	10.5	5.3
$S_{P/X}$ / mg g^{-1}	2.7	1.3	2.9	2	2.5	1.5
L_V / mg L^{-1} h^{-1}	0.2	0.05	0.27	0.07	0.2	0.1

Table 11 Results obtained with media GGM/L – GGM/P.

Batch	L	M	N	O	P
Amino acids	Gly, Ile	Gly, Phe	Ile, Met	Ile, Phe	Met, Phe
X / g L^{-1}	4.1	2.5	2.9	3.5	3.7
P / mg L^{-1}	14.4	6.8	3.5	8.8	5.6
$S_{P/X}$ / mg g^{-1}	3.5	2.7	1.2	2.5	1.5
L_V / mg L^{-1} h^{-1}	0.24	0.1	0.1	0.2	0.1

Table 12 Results obtained with media GGM/Q – GGM/V.

Batch	Q	R	S	T	U	V
Amino acid	Met	Ile	Gln	Ala	Gly	Phe
X / g L^{-1}	3.7	3.5	3.8	1.6	3	3.6
P / mg L^{-1}	1.1	3.2	1.5	2	3.9	5.8
$S_{P/X}$ / mg g^{-1}	0.3	0.9	0.4	1.3	1.3	1.6
L_V / mg L^{-1} h^{-1}	0.03	0.06	0.03	0.04	0.09	0.1

The results showed that amino acid supplementation had different effects on the outcome of the cultivations. In batches B, C, D, H, J, and L biomass concentration increased above average (almost factor 2) while in batches A, F, I, and T it was well beyond average (factor 0.5). How the different amino acid combinations caused the differences in biomass concentration and product formation, could only be speculated. However, growth and product formation was not only determined by the nutrients in the media but also by factors like stress, metabolic burden, and growth rate. Especially in the case of plasmid production it has been hypothesized that plasmid replication is strongly correlated with growth rate. At rather accelerated growth the product selectivity dropped during cultivation while at moderate growth rates it remained constant. Nevertheless, this rather simple image has not been verified and it can be assumed that replication control is more complicated than anticipated.

Highest productivity could be obtained in batches H (Ala, Ile) and L (Gly, Ile). Therefore these media were chosen for bioreactor cultivation. Batch cultivations with medium GGM/L showed a maximum biomass concentration of X = 4.1 g L^{-1} after 24 h cultivation. Maximum selectivity $S_{P/X}$ = 2.9 mg g^{-1} was achieved after 16 h while highest product concentration was obtained after 22 h of cultivation with P = 7.7 mg L^{-1}. Productivity was calculated to L_V = 0.4 mg L^{-1} h^{-1}. The course of the cultivation is shown in Figure 15.

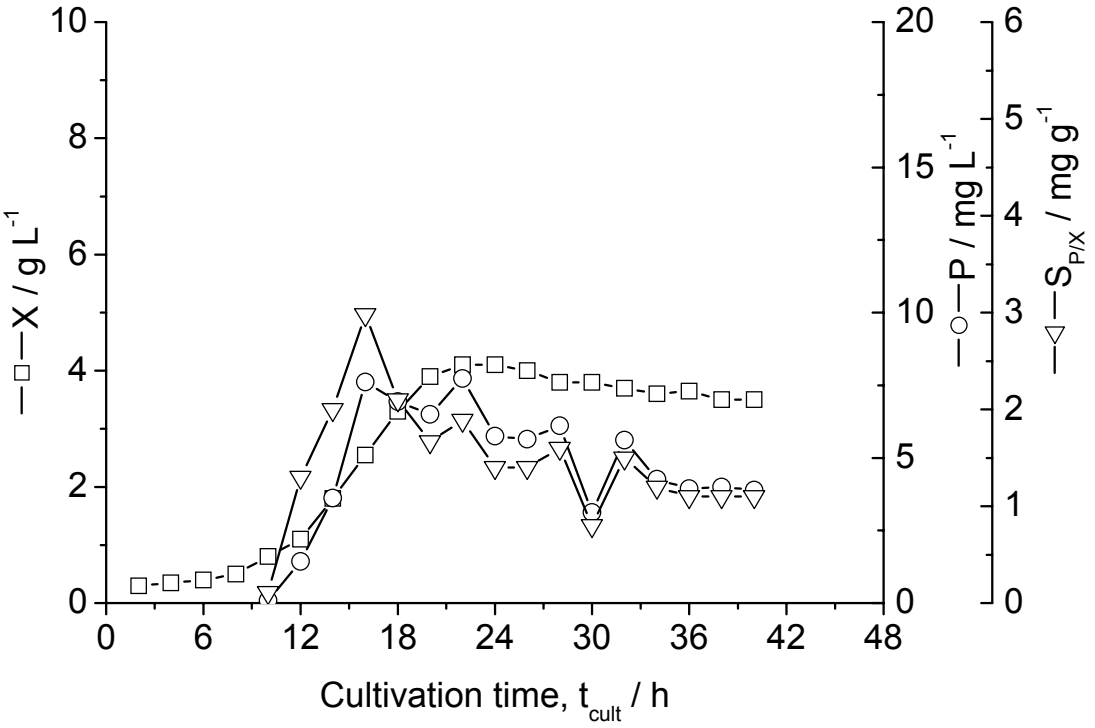


Figure 15 Bioreactor cultivation using GGM/L medium containing glycine and isoleucine.

The results showed that the product concentration decreased by a factor of almost 2 during the stationary phase. Such a product loss was not observed in cultivations with the semidefined HSG medium and was rather a phenomenon that was associated with defined media. Analysis of the medium composition during cultivation showed that growth was limited by the depletion of glycerol after 26 h of cultivation. Acetic acid did not reach inhibitory concentrations.

Batch cultivation on GGM/H medium resulted in a biomass concentration of $X = 4.8\ g\ L^{-1}$, product concentration of $P = 43\ mg\ L^{-1}$, and a selectivity of $S_{P/X} = 8.9\ mg\ g^{-1}$ as shown in Figure 16.

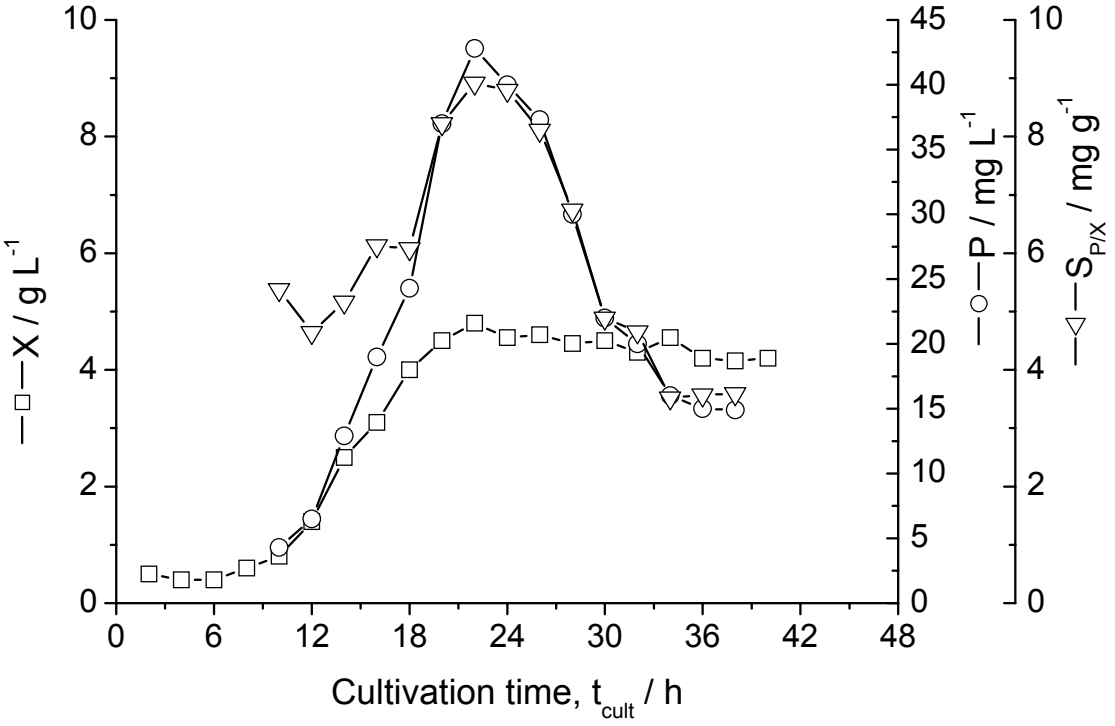


Figure 16 Bioreactor cultivation on GGM/H medium containing alanine and isoleucine. Scale of right axes for P and $S_{P/X}$ was changed in comparison to the preceding figures.

Although a maximum product concentration was achieved at the beginning of the stationary phase it decreased dramatically during the stationary phase and reached values of $P = 15\ mg\ L^{-1}$. Nevertheless, the selectivity after 38 h of cultivation was with $S_{P/X} = 3.6\ mg\ g^{-1}$ still comparable to the values obtained in HSG medium. It should be noted that the selectivities in GGM/H and GGM/L shaking flask cultures were almost comparable but significantly different during bioreactor cultivations. At this moment, it could only be hypothesized that other factors might have influenced the product formation during bioreactor cultivation.

In conclusion, the results obtained for the supplementation of a basic glycerol medium (GGM) with amino acids showed that an increase in productivity and selectivity was

possible. This increase could be refered to the increased product concentration in these media. The basic glycerol medium supplemented with L-isoleucine and L-alanine showed final product concentrations that were comparable or higher to those produced in HSG medium. However, the rather erratic selectivity and the large product loss observed during the stationary phase questioned the general application of this medium for pharmaceutical plasmid DNA production. In addition, it was not answered in which way amino acid supplementation affected plasmid production and if other factors were also involved. Because the optimization of a medium composition resulted in more questions than clues, it was considered necessary to analyze the cultivation of plasmid producing *E. coli* on defined media in more details.

4.1.3 Influence of Inoculation Cultures on Plasmid Production

Further studies were limited to the differences in selectivity between GGM and HSG medium. The influence of the inoculation culture was one of the first parameters that should be taken into account. For this purpose batch cultivations in a 2.5 L bioreactor have been conducted. A closer look was also put on the beginning of the cultivation which was more or less disregarded in earlier research. The reference cultivation on HSG medium was analyzed under these considerations. An inoculation culture was prepared using HSG medium for a period of 8 hours. The inoculation volume was chosen in that way that the OD_{600} in the main culture would be 0.05. Samples were taken in regular intervals and analyzed as before. The results are presented in Figure 17.

The plasmid concentration and selectivity at inoculation was determined from the inoculation culture. Due to the low biomass concentrations during the first hours of cultivations the selectivity at this time might be more error prone in comparison to values obtained later. Nevertheless, a tendency could be observed here. The inoculation culture showed a selectivity about 3.2 mg g^{-1} which fell in the first four hours of main cultivation to a value of 1.9 mg g^{-1}. In this period a high specific growth rate was observed which fell slightly from 0.5 h^{-1} to values below 0.4 h^{-1}. When lower specific growth rates were reached the selectivity rises again to $S_{P/X}$ = 3.9 mg g^{-1} and reaches values of 3.6 mg g^{-1} after 13 hours when the culture entered the stationary phase. Biomass concentrations reached values of 7 g L^{-1} being typical for cultivations of plasmid bearing *E. coli* DH5α on HSG medium.

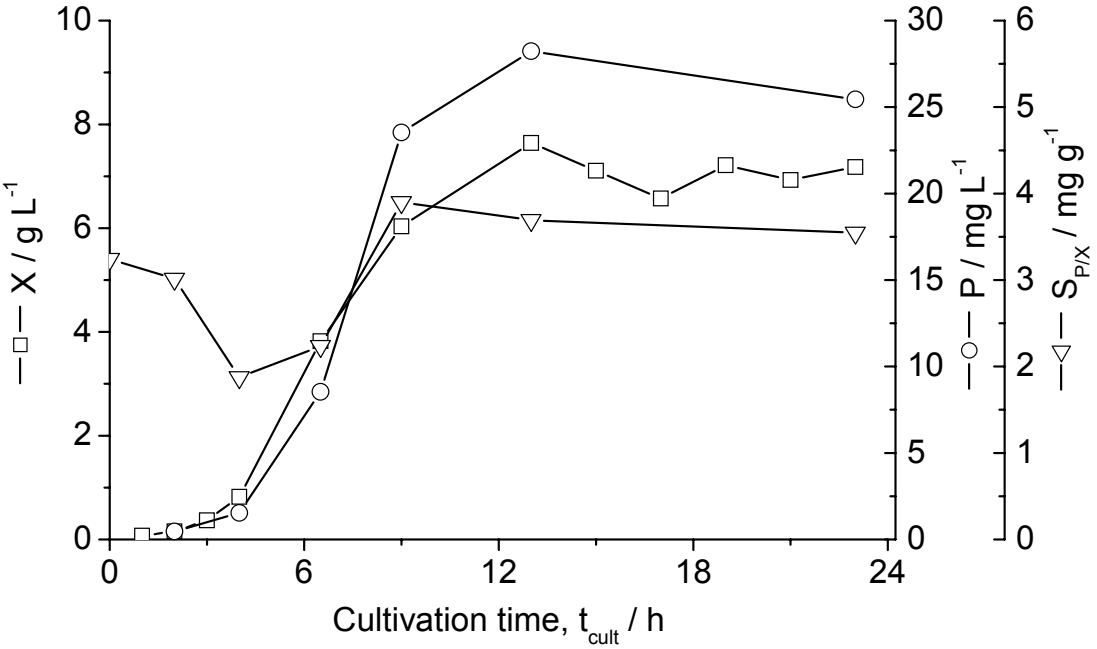


Figure 17 Cultivation on HSG medium using a HSG based inoculation culture. Scale of P-axis was changed.

The cultivation in basic glycerol medium was performed under the same conditions as the cultivations in HSG medium mentioned before. The inoculation culture had an optical density of OD_{600} = 3.5 after 7 hours and was used to generate an initial OD_{600} of 0.05 in the GGM bioreactor cultivation. The results obtained from this cultivation are summarized in Figure 18.

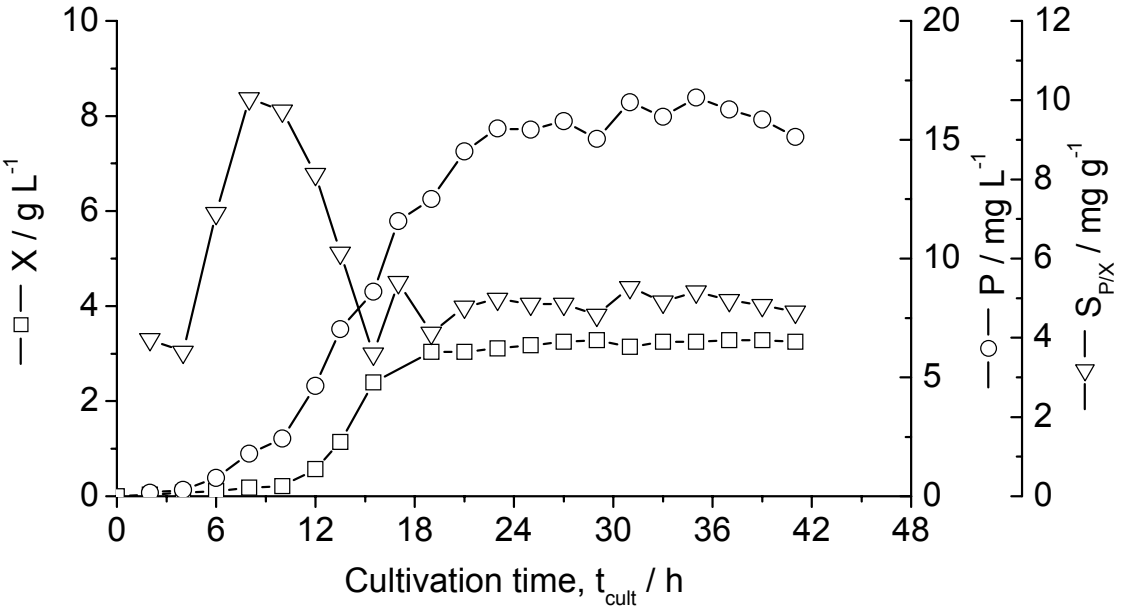


Figure 18 Cultivation on GGM medium using an HSG based inoculation culture.

The first observation was that the cultivation in the defined medium took longer because growth was slower in comparison to cultivations on HSG medium. The specific growth rate varied between 0.3 h^{-1} and 0.1 h^{-1} and the culture reached the stationary phase after 20 hours. The biomass concentration was with X = 3 g L^{-1} lower than the values obtained in HSG culivations because the extract concentrations had not been compensated by additional glycerol as carbon source. The yield coefficient $Y_{X/S}$ has been calculated to be 0.10 with respect to 33 g L^{-1} of media components in total. In comparison, in HSG medium $Y_{X/S}$ was 0.18 (relative to 42 g L^{-1} media components) which was still low but typical for plasmid cultivations. Due to the low biomass concentration the plasmid concentration was equally low with P = 15 mg L^{-1} during the stationary phase. In comparison to other cultivations on defined glycerol media the plasmid concentration remained considerably stable during stationary phase and no product loss was observed. Another striking observation was the increase of selectivity during an early period of the cultivation at the beginning of the growth phase to values of 10 mg g^{-1}. When entering the exponential growth phase the specific growth rate increased and selectivity fell to values between 4 and 5 mg g^{-1} at the end of growth and during stationary phase. Repetition of this cultivations showed the same results. The change in selectivity during this cultivation differed from the development of this parameter in HSG cultivations. One possible

explanation might have been that a medium component originating from HSG inoculation was a limiting factor and thus was responsible for the development of the selectivity here. However, this explanation was excluded because cells taken from a shaking flask culture on HSG medium have been separated by centrifugation, resuspended in GGM medium and then used for the inoculation of a main culture. This main culture also showed a comparably high selectivity. Therefore, the changes in the selectivity should be a result of the physiological state of the cells due to the medium change from inoculation to main culture. To further verify this hypothesis an inoculation culture was prepared using GGM medium. The results of the bioreactor cultivation are shown in Figure 19.

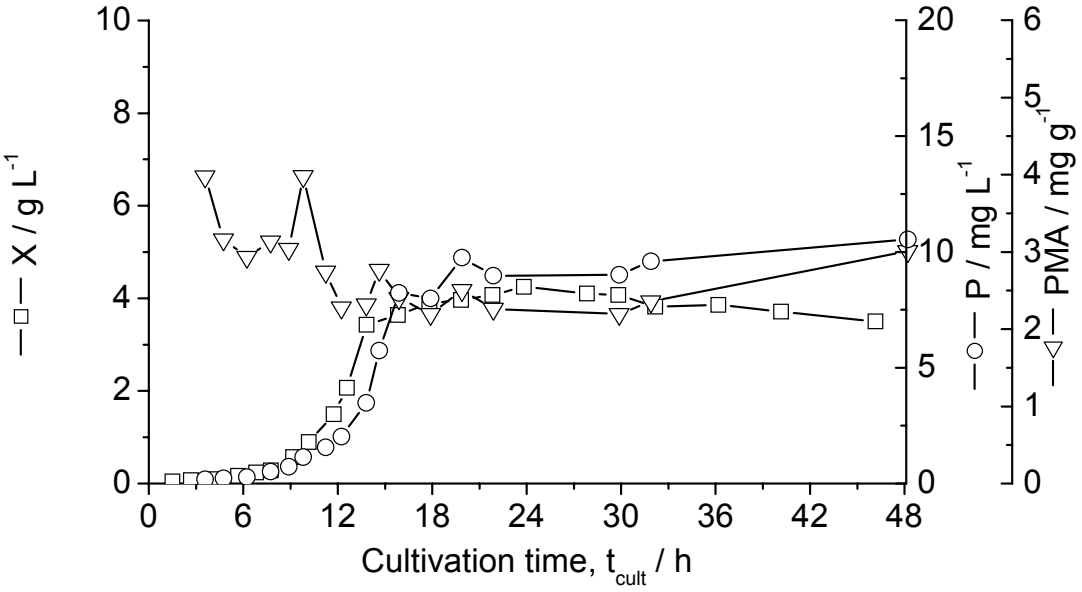


Figure 19 Cultivation on GGM medium with an inoculation culture grown on GGM.

In this case the selectivity was under 4 mg g^{-1} during lag and early growth phase and dropped to values below 3 mg g^{-1} at the end of the growth period, remaining almost constant during stationary phase. In comparison to the previously described cultivation the lag phase was shorter when GGM was used for the inoculation culture instead of HSG. The cells have already adapted to the new medium and growth resumed very quickly. An increase of the selectivity that was observed with HSG medium for the in-

oculation culture could not be seen here. During growth the specific growth rate increased steadily which could explain the decreasing selectivity during this period.

For further studies it was decided to extend the growth phase during cultivation so that effects associated with changes in growth rate could be identified better. Therefore, a more concentrated glycerol medium was used for further experiments. In this medium the concentrations of all components were increased as shown in Table 13.

Table 13 Composition of concentrated GGM (k-GGM) medium in comparison to GGM.

Compound	GGM	k-GGM
Glycerol	14.9 g L^{-1}	29.8 g L^{-1}
KH_2PO_4	1.5 g L^{-1}	3 g L^{-1}
K_2HPO_4	2.3 g L^{-1}	4.6 g L^{-1}
NaCl	2.5 g L^{-1}	2.5 g L^{-1}
Na_3citrate · 5.5 H_2O	9.5 g L^{-1}	9.5 g L^{-1}
$MgSO_4$ · 7 H_2O	0.25 g L^{-1}	0.75 g L^{-1}
$(NH_4)_2SO_4$	2.5 g L^{-1}	10 g L^{-1}
Trace element solution	2 mL L^{-1}	2 mL L^{-1}
Thiamine · HCl	11.2 mg L^{-1}	16.8 mg L^{-1}

This concentrated medium (k-GGM) was inoculated with a culture prepared on GGM that reached an OD_{600} of 2.5 and was in the early growth phase. The results obtained from this cultivation are summarized in Figure 20 and Figure 21.

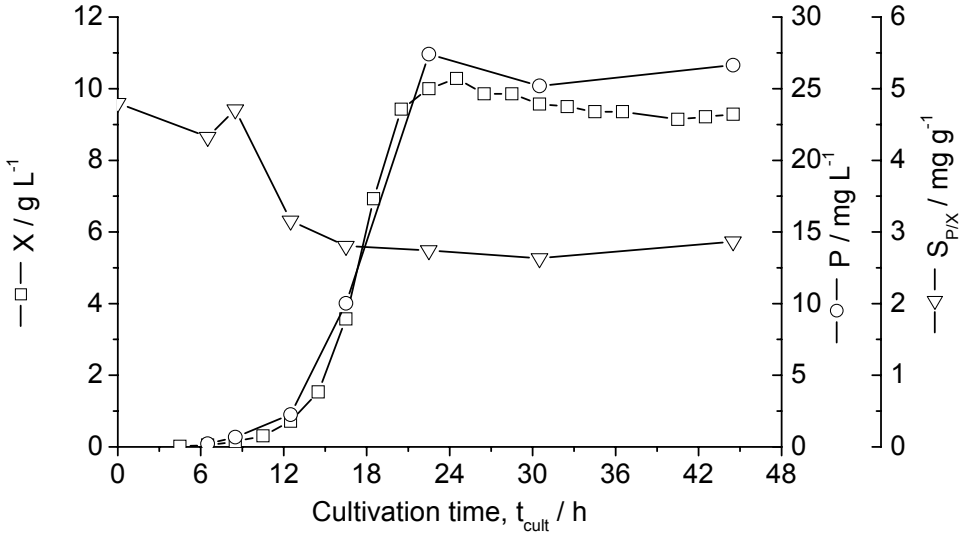


Figure 20 Bioreactor culture grown on k-GGM inoculated with a GGM culture with an OD_{600} of 2.5. Scales of X- and P-axes have been changed.

Figure 20 shows the development of biomass concentration X, plasmid concentration P, and selectivity $S_{P/X}$ over 45 hours of cultivation while Figure 21 shows the consumption of glycerol and ammonia as well as the formation of acetic acid in relation to cell growth. Biomass concentration reached its maximum value at the beginning of the stationary phase after 24 hours of cultivation with X = 10 g L^{-1}. This is even higher than the biomass concentration achieved with HSG medium. Additionally the yield coefficient $Y_{X/S}$ has increased in comparison to the cultivation in unconcentrated GGM. This could be explained by a more balanced nutrition due to the increased concentration of ammonium sulfate (factor 4) in comparison to glycerol (factor 2). As shown in Figure 21 both components were digested equally fast by *E. coli* and their depletion corresponded with the culture reaching the stationary phase.

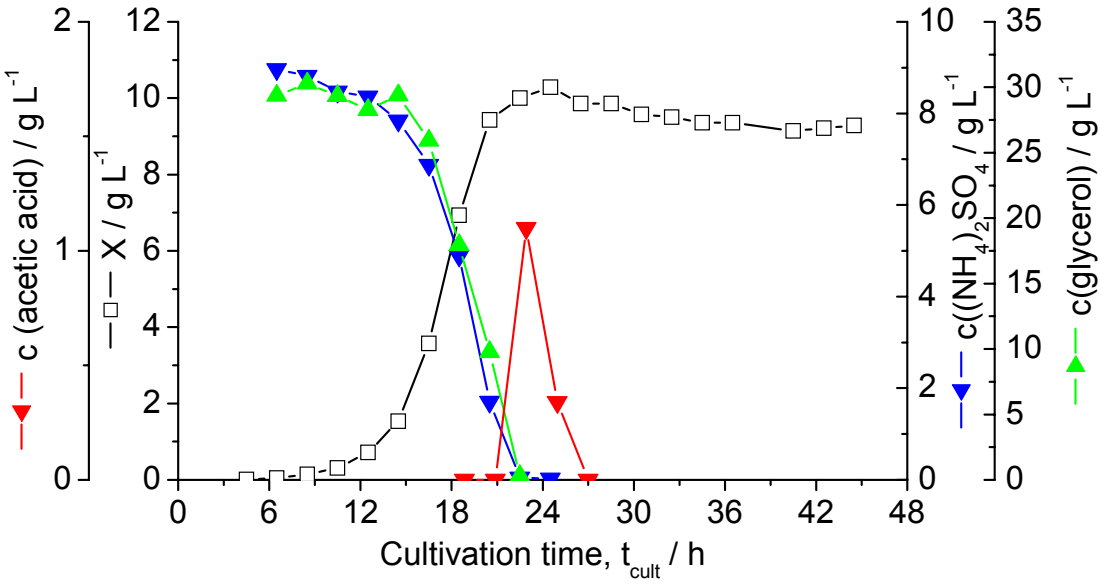


Figure 21 Utilization of medium components and acetate formation during the cultivation presented in Figure 20.

Plasmid concentration reached values of P = 27.4 mg L^{-1} which was comparable to values obtained in cultivations with HSG medium while the selectivity was slightly lower with $S_{P/X}$ = 2.7 mg g^{-1} when entering the stationary phase. Another interesting observation was found in the fact that the selectivity showed rather high values of 5 mg g^{-1} right after inoculation which fell at high specific growth rates (0.6 h^{-1}) to the final value of 2.7 mg g^{-1} which remained constant afterwards at a specific growth rate around 0.4 h^{-1}.

In the following an inoculation culture on GGM that has reached an OD_{600} of 11 was applied. This culture was in the late exponential growth phase and it was assumed that growth in the main culture would resume very fast. The concentrated GGM medium was inoculated to an initial OD_{600} of 0.05. The development of the cultivation is shown in Figure 22. Biomass concentration reached a value of X = 10 g L^{-1} while the plasmid concentration increased only to 13 mg L^{-1} which was significantly lower than the values obtained in the cultivation before. The selectivity of the inoculation culture was found to be low with 1.3 mg g^{-1}. This low selectivity did not change during main batch cultivation and no further plasmid loss was observed although it might have been expected that at higher specific growth rates the average plasmid copy number might also fall in the same manner as it did in the preceding cultivation.

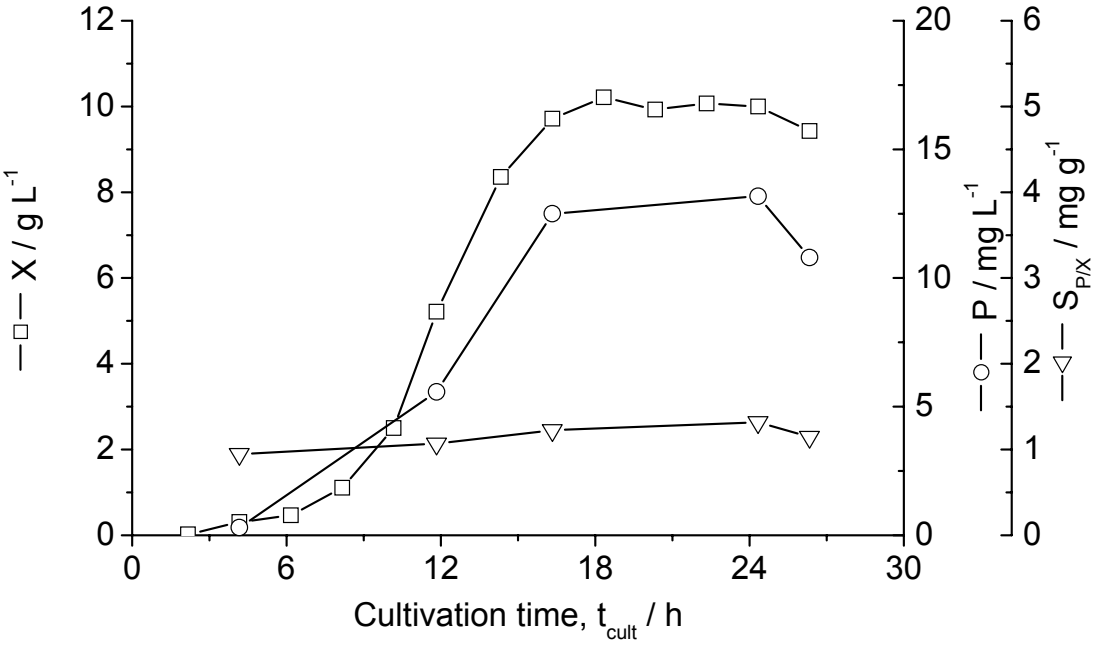


Figure 22 Cultivation on k-GGM medium using a GGM inoculation culture with an OD_{600} of 11.

By now it was obvious that the inoculation culture had a significant impact on the development of the main batch culture. Although not interesting for production but certainly worth for getting a more profound understanding of plasmid replication during cultivation, it was decided to inoculate HSG medium with GGM cultures. In the first cultivation the inoculation culture was incubated until an OD_{600} of 2.4 was reached and HSG medium was inoculated to an initial OD_{600} of 0.05. The development of this culture is presented in Figure 23. The values obtained for biomass concentration were once again typical for cultivations on this medium and reached a final level of 8.4 g L^{-1} which was about 0.6 to 0.7 g L^{-1} higher in comparison to the HSG cultivations described before. The specific growth rates were considered to be equally fast during the early growth period and fell to lower levels during the second half of exponential growth. The main difference to other HSG cultivations was found in the plasmid concentrations. Here, we observed a maximum concentration of 10 mg L^{-1} after 12 hours growth which was less than half the concentration obtained when using a HSG based inoculation culture. The development of the selectivity in this cultivation was very interesting, too. The initial value was calculated from the inoculation culture and was found to be comparably high with 4.4 mg g^{-1}. This was in good agreement with the results obtained from

other inoculation cultures under these conditions. With the high specific growth rates observed in the early cultivation period the selectivity dropped to a minimum value of 0.6 mg g^{-1} and increased again when the specific growth rate was lower and reached its final value of 1.2 mg g^{-1} during stationary phase.

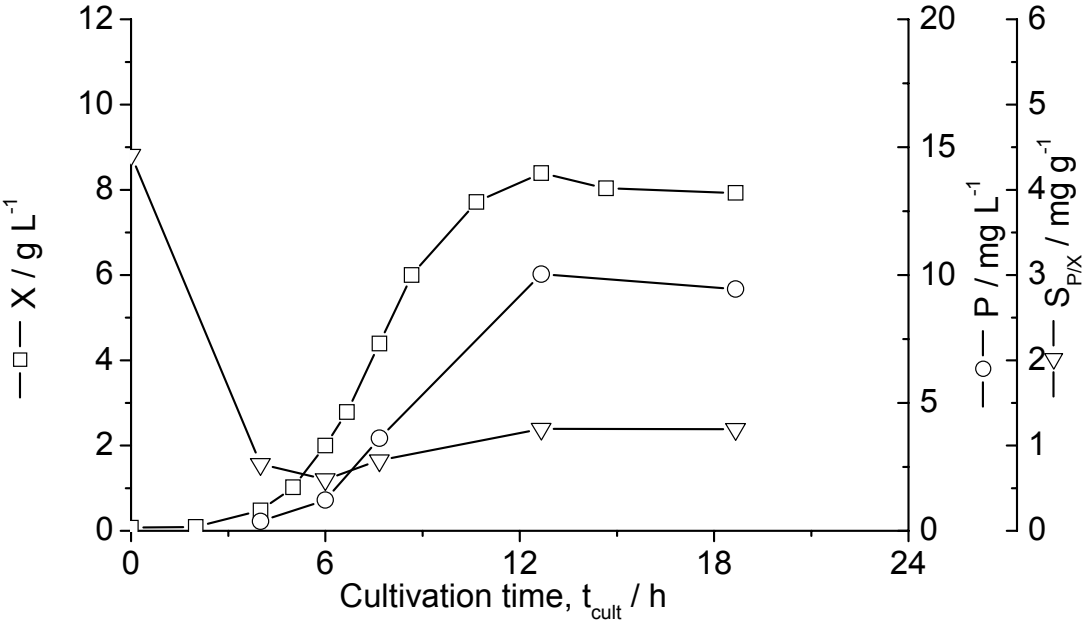


Figure 23 Cultivation on HSG medium after inoculation with a GGM culture at OD_{600} = 2.5.

When an inoculation culture on GGM with on OD_{600} of 11 was used, an initial selectivity of 1.1 mg g^{-1} was observed which did not increase significantly during the second half of the growth period as shown in Figure 24. Additionally, a segregational instability was noted during these cultivations. It could be concluded that a larger amount of plasmid free cells developed in the early period of this cultivations, thus the culture could not recover from that later and only a minor increase in selectivity was observed during the later growth period. Furthermore, an increased biomass concentration of X = 9.1 g L^{-1} was measured which could be explained by the lower energy consumption of the cells with low plasmid copy number.

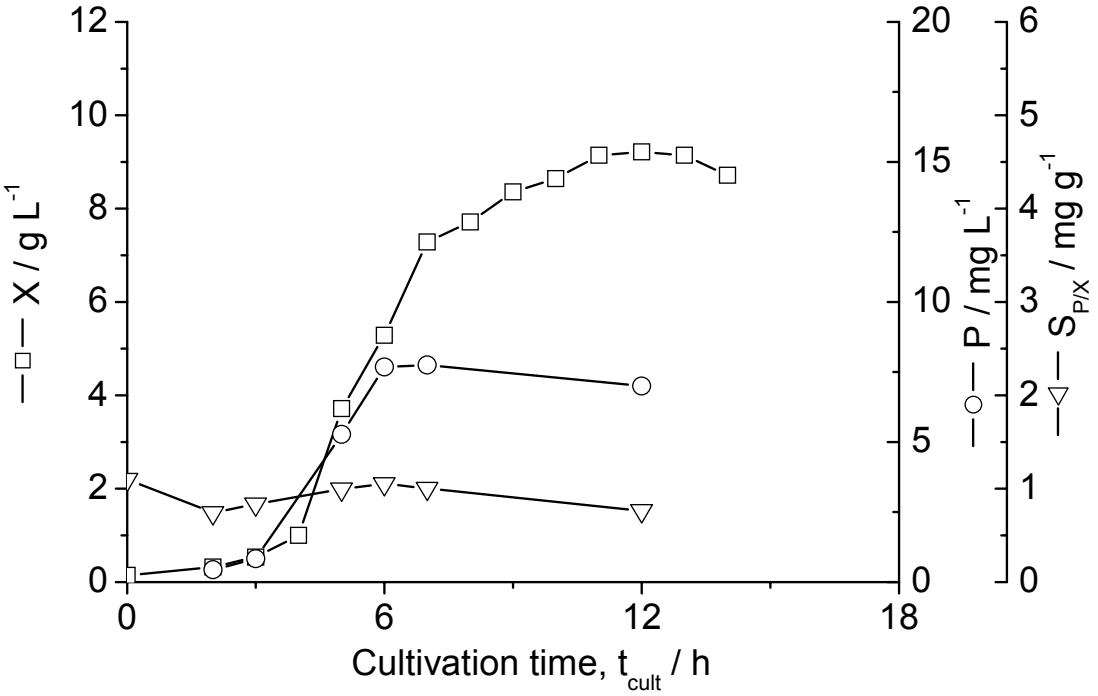


Figure 24 Cultivation on HSG medium after inoculation with a culture grown on GGM to an OD_{600} of 11.

The results from these experiments are summarized in Table 14 and compared to values described by Schmidt, Bär, and Voß (Bär, 2005; Schmidt, 1998; Voß, 2001; Voss *et al.*, 2004). Higher yield coefficient $Y_{X/S}$ have been achieved with k-GGM in comparison to GGM medium which could be explained by the increased concentration of ammonium and magnesium. The values obtained were comparable to the yield coefficients determined for HSG medium and the amino acid supplemented medium GGM/H. However, the results also showed that selectivities could differ substantially although yield coefficients ($Y_{X/S}$) had comparable values. This indicated that the physiological conditions of *E. coli* in the inoculation culture significantly influenced product formation during main bioreactor cultivation.

Wih respect to productivities it should be noted, that the calculation did not take preparation of media and bioreators as well as subsequent cleaning into account. These operations are more time consuming for cultivations on defined media.

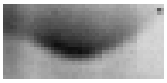
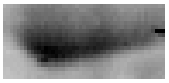
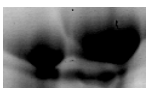
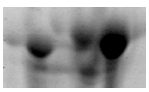
Table 14 Comparison of different cultivations for plasmid production in batch operation. The values for $S_{P/X}$ represent the maximum selectivity observed in the bioreactor cultivation.

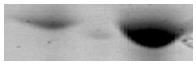
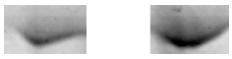
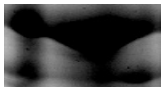
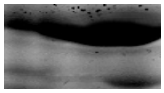
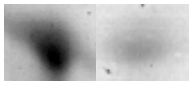
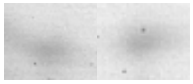
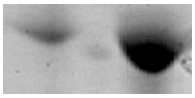
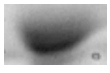
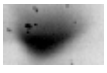
Medium (Inoculum)	X_{max} / g L^{-1}	P / mg L^{-1}	$S_{P/X}$ / mg g^{-1}	$Y_{X/S}$ / -	L_V / mg L^{-1} h^{-1}	$S_{P/X}$ (Inoc) / mg g^{-1}	Reference
HSG (HSG)	7.6	26	3.4	0.18	1.4	n/a	Schmidt, 1998
M9 (HSG)	3.6	4.7	1.3	0.20	0.11	n/a	Voß, 2001
HSG+ (HSG)	23.9	45	2.0	0.41	2.3	n/a	
Glyerol Glutamate Medium (HSG)	20	50	2.5	0.21	1.25	n/a	Voß *et al.*, 2004
GGM (HSG)	3.2	12	5	0.11	0.4	n/a	Bär, 2005
GGM/H (HSG)	4.8	42.8	8.9	0.16	1.95	n/a	
HSG (HSG)	7.7	25.2	3.5	0.18	2.5	n/a	Fischer, 2008
HSG (GGM OD_{600} = 11)	9.2	7.7	1.1	0.22	1.1	1.3	
HSG (GGM OD_{600} = 2.5)	8.4	10.0	1.2	0.20	0.79	4.4	
GGM (HSG)	3.3	14.5	5.0	0.10	0.69	n/a	
GGM (GGM)	4.2	8.2	2.4	0.13	0.59	4.5	
k-GGM (GGM OD_{600} = 2.5)	10.0	27.4	2.7	0.17	1.2	4.8	
k-GGM (GGM OD_{600} = 11)	10.4	12.5	1.2	0.18	0.68	1.3	

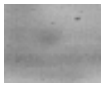
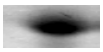
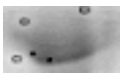
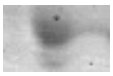
The influence of the physiological condition in the inoculation culture did not explain all the results obtained. During cultivations in defined glycerol media a product loss has been observed during the late growth phase and in exponential phase. One explanation might be that the cells already showed a stringent response due to stress factors or nutrient limitation.

To analyse this theory, samples taken from the batch cultivations at a defined biomass concentration (OD_{600} = 3) during exponential growth were analyzed by 2D gel electrophoresis and spots that were significantly different in cultivations on GGM and HSG were then picked and identified by MALDI/TOF analysis and MASCOT database. The results obtained from these experiments are summarized in Table 15.

Table 15 Comparison of protein expression during batch reactor cultivation on HSG and GGM medium with respect to selected protein spots from 2D gel electrophoresis.

Class	HSG	GGM	Gene	Protein name	Regulation in GGM
AA-metabolism[1]			hisD	Histidinol-DH	+
AA-metabolism			argG	Argininosuccinate synthase	+
AA-metabolism			leuC, leuD	Subunit of isopropylmalate isomerase	-
AA-metabolism			glyA	Serine hydroxymethyl transferase	-
AA-metabolism			aspC	Aspartate aminotransferase	+
HS/KS			mopA	Part of chaperon system GroEL (Hsp60)	-
HS/KS			dksA	Dnak repressor protein	=

Class	HSG	GGM	Gene	Protein name	Regulation in GGM
C-metabolism[3]			gatY	Subunit of tagatose-1,6-bisphosphate aldolase	+
C-metabolism			ybhE	6-Phospho-gluconolactonase	=
C-metabolism			mdh	Malate-DH	+
Nk-metabolism[4]			guab	IMP-DH	-
Nk-metabolism			dut	Desoxyuridin triphosphatase (dUTPase)	=
Protein Synthesis			rpoA	Subunit of RNA-polymerase *core* enzyme	=
Protein Synthesis			tufA, B	Elongation factor Tu (EF-Tu) subunits	-
Protein Synthesis			rplL	50S Ribosomal subunits	-
Protein Synthesis			tsf	Elongation factor EF-Ts	-
Protein Synthesis			rpsF	Component of 30S subunit of the ribosomes	-
Transport			proX	Subunit of proline ABC transporter	+
Transport			dppA	Subunit of dipeptide ABC transporter	+
Transport			rbsB	Subunit of ribose ABC transporter	+

Class	HSG	GGM	Gene	Protein name	Regulation in GGM
Transport			livK, livJ	Subunit of ABC transporter for leucine, isoleucine and valine (branched AA)	+
Transport			glnH	Glutamine ABC transporter	+
Misc			ppa	Inorganic pyrophosphatase	-
Misc			osmY	Hyperosmotically induced protein	+
Misc	 	 	atpA, atpD	AtpA/D = ATP-Synthase, F1 complex, α/β subunit	-

[1] Amino acid metabolism
[2] Heat/Cryo shock
[3] Carbon metabolism
[4] Nucleotide metabolism

Because GGM medium was not supplemented with amino acids, differences in the proteome of the amino acid metabolism could be expected. Enzymes like ArgD and HisD that are important in the *de novo* synthesis of amino acids were overexpressed during cultivations on GGM medium. GlyA was overexpressed in HSG medium. This enzyme plays a key role in the biosynthesis of purines, thymidine, and fatty acids. It is also mandatory for growth on glycerol (Joyce *et al.*, 2006). The same applies for the overexpression of SerC.

All proteins that are part of an ABC transporter were overexpressed during cultivation on GGM medium. This could indicate a nutrient limitation. Also interesting was the overexpression of a ribose ABC transporter (RbsB) because ribose is used for nucleotide synthesis. *De novo* synthesis of amino acids could lead to an overload of the citric acid cycle and the anaplerotic pathways. In this way, insufficient amounts of pentose

sugars are synthesized for nucleotide synthesis during gluconeogenesis. However, adding ribose to the medium may risk accelarated growth because this sugar is metabolized very quickly. Furthermore, a significant overexpression of the ABC transporter LivFGHMJ for leucin, isoleucine, and valine was observed, as well as an overexpression of a subunit of ProX. This transporter does also have an affinity to glycine betaine that serves as an osmoprotectant in *E. coli* (Lucht and Bremer, 1994). Additionally, OsmY as a hyperosmotically induced protein, was found to be overexpressed during cultivation in GGM medium (data not shown) indicating a reaction to increased osmolarity in the medium. Osmotic stress can induce the stringent response. For this reason, the addition of glycin betaine or its precursor choline, or proline might be reasonable. Furthermore, a stringent response was indicated during cultivation on GGM medium by the decreased concentration of IMP dehydrogenase (GuaB) which is necessary for the formation of GMP (Cashel *et al.*, 1996). Another important characteristic of the stringent response was the low expression of elongation factors indicating a restricted protein biosynthesis. Additionally, the ribosome number seemed to be reduced due to the low expression of 50S and 30S ribosomal subunits. The stringent response is also associated with a reduced synthesis of RNA and could be a reason for reduced plasmid replication during growth on defined GGM medium.

4.1.4 Conclusions

The results showed that a simple correlation of plasmid replication and reduced specific growth rate could not explain the low plasmid concentrations obtained in cultivations on defined media. The experiments performed by Bär (2005) indicated that amino acid supplementation could counteract some of the adverse effects of nutrient limitation and an increased selectivity for plasmid DNA was observed although it could not be determined how the amino acids influenced plasmid selectivity and if other factors were involved. The research was continued by Fischer (2008) who could outline that the physiological state of the cells in the inoculation culture had a profound impact on plasmid replication in the main bioreactor cultivation. In addition, first results obtained from proteome analysis indicated a stringent response during these cultivations which is currently verified in ongoing research. In conclusion, the results obtained clearly showed that cultivation processes could be optimized by simple screening of media

components. It is obvious, that this is generally a rather tedious and time consuming strategy. A more interesting insight into product formation during batch reactor cultivation could be achieved by proteome analysis. In this way, not only the effects of media components on growth and product formation could be monitored but also the effects of culture conditions and other factors that influenced the physiological state of *E. coli*.

In this way, the aim of this research was not just to develop a suitable cultivation process but to get a more detailed insight into the factors influencing plasmid replication under conditions found in bioreactor cultivation.

It should be noted, that higher product amounts could be gained by the application of fed batch strategies that was already outlined in the Theoretical Background. However, the development of a fed batch strategy using a defined medium is only considered to be successful when it is supported by a profound understanding of the factors influencing replication.

4.2 Scale Up of Alkaline Lysis

The disruption of large amounts of biomass is a prerequisite for large scale plasmid manufacturing. As already outlined, a simple scale up in a stirred reactor is afflicted with product loss due to extreme pH. Additionally, extensive stirring can break the large genomic DNA of *E. coli* into smaller fragments with a rather broad size distribution and thus contaminate the product stream. Gentle lysis by continuous mixing of small volumes of biomass suspensions with alkaline lysis buffer can avoid the problems described above. Nevertheless, cell debris precipitated along with proteins and genomic DNA after neutralization has to be removed equally careful. Common procedures like centrifugation and filtration are rather tedious for this purpose. In case of filtration the precipitate blocks the filter material after a short time already.

4.2.1 Continuous Lysis and Froth Flotation

To solve this problem we integrated a flotation column into a continuous lysis procedure. Cell suspension (200 g wet cell paste in 2 L lysis buffer) and alkaline lysis buffer (0.2 M NaOH, 1 % SDS; 2 L) were mixed using a simple T-connector where the mixture left on a 90° angle. Lysis took place in the subsequent flow volume and residence time was optimized for complete lysis as described in Voss *et al.* (2005). In this way, pressure drops occuring in static mixers (Wan *et al.*, 1998) could be avoided. Neutralization and separation from the debris was achieved using simple froth flotation by sparging air through a sintered metal plate at the bottom of the column into the neutralization buffer (3.0 M KOAc, pH 5.5 adjusted by the addition of acetic acid; 2 L) as shown in Figure 25. The diameter of the flotation column was 22 cm while the sintered metal plate had a diameter of 20 cm.

The high clarity achieved by this technique becomes already obvious from the picture on the right. Additionally, the clarity was verified by measuring the optical density at 600 nm (OD_{600}) and compared to lysates obtained by a classical, “manual” lysis. For this manual lysis, 30 mg wet cell paste was suspended in 300 µL Tris-EDTA buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0) until a homogeneous suspension was achieved. A volume of 300 µL of alkaline lysis buffer was added and mixed by inverting for 5 minutes. Subsequently the solution was neutralized by the addition of potassium acetate

buffer and mixed by inverting for 5 minutes. Clarification was achieved by centrifugation at 9800 rpm for 10 minutes and subsequent filtration. The liquid obtained after flotation showed an OD_{600} of 0.002 while a lysate obtained from the lysis procedure described before had an OD_{600} of 0.07 after centrifugation. Subsequent filtration clarified this lysate further and an OD_{600} of 0.03 was achieved.

For efficient cell disruption two parameters had to be optimized. First a suitable flow rate had to be used for efficient mixing of cell suspension and alkaline solution. Secondly the residence time in the flow volume after mixing had to be sufficient for complete lysis.

Figure 25 Scale up of alkaline lysis and neutralisation using a flotation column.

For this purpose cells were mixed at different flow rates and alkaline lysis was achieved in a 3 m long tube set after the T-connector. The concentration of plasmid DNA found in the lysates was determined after concentration using a QIAGEN tip-20 anion exchange column. These values were compared to a “manual” lysate where mixing was achieved by inverting as already described before (Table 16).

Table 16 Plasmid concentration in the neutralized lysate with respect to flow rate in continuous operation in relation to manual lysis.

Flow rate / cm min^{-1}	Relative plasmid concentration / %
manual	100
280	86
550	84
830	78
900	99
1080	63
1400	38

Flow rates between 700 and 900 cm min^{-1} were found to be optimal because efficient mixing was achieved and the residence time in the following tube was between 20 and 40 s resulting in complete cell disruption. Additionally, plasmid DNA was found to be unaffected by the conditions applied during continuous lysis regarding form distribution as can be seen in Figure 26. A densitometric analysis of the agarose gel showed that the ccc-oc ratio did not change when using increased flow rates for the continuous lysis. In addition, the densitometric analysis also confirmed the relative concentrations that have already been outlined in Table 16. Furthermore, the structural stability of the 4.6 kb plasmid in this experiments could already been concluded from the results described by Levy *et al.* (2000) showing that plasmids with a size of 13 kb or lower are not broken under defined shear.

Because plasmid concentration in the biomass used for these experiments could vary from cultivation to cultivation, only relative concentrations were determined. Nevertheless, a comparison of the amount of plasmid DNA isolated by a classical lysis procedure and the continuous lysis/froth flotation operation is interesting. The results obtained by Clemson and Kelly (2003) showed, that an increased concentration of biomass in the suspension did not increase the product concentration significantly. On the contrary, an

increased viscosity was observed making subsequent procedures more sensitive to shear.

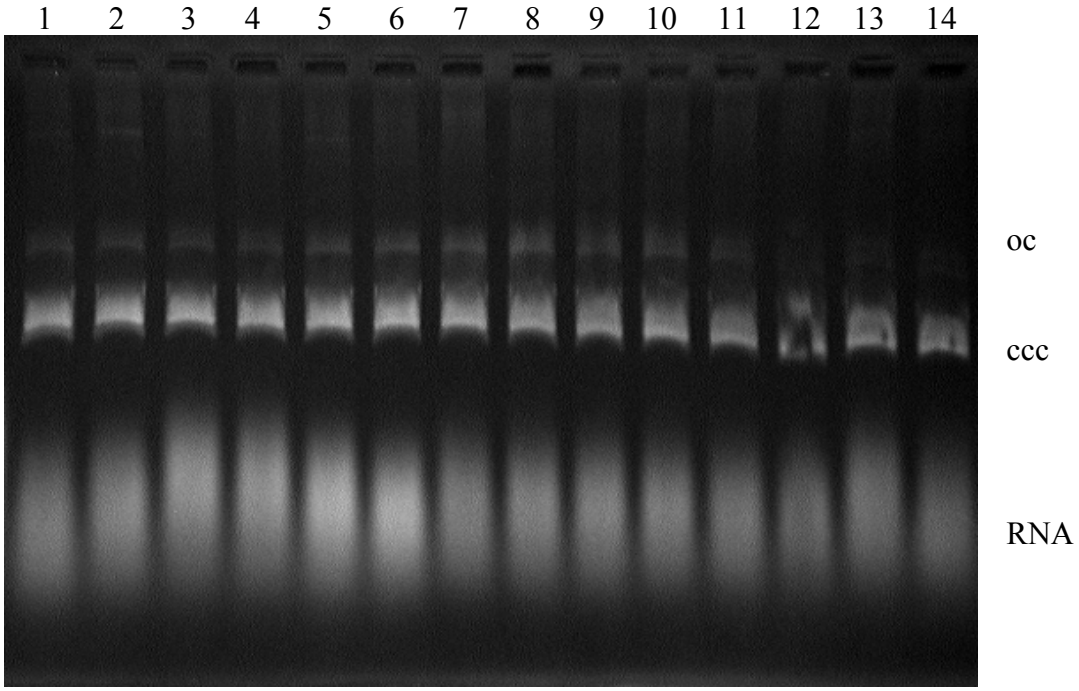


Figure 26 Agarose gel electrophoresis of the lysates obtained from continuous alkaline lysis at different flow rates. Lanes 1, 2: Manual lysis. Lanes 3, 4: 280 cm min^{-1}. Lanes 5, 6: 550 cm min^{-1}. Lanes 7, 8: 830 cm min^{-1}. Lanes 9, 10: 900 cm min^{-1}. Lanes 11, 12: 1080 cm min^{-1}. Lanes 13, 14: 1400 cm min^{-1}.

For this reason, the biomass concentration in the suspension was 30 g of wet cell paste in 3 L buffer. When defined conditions were used for cell harvesting after cultivation, a good correlation between the weight of the wet cell paste and of the dry biomass could be observed. In our case a factor of 0.3 could be applied corresponding to 10 g dry biomass. Taking an average selectivity of 3 mg g^{-1} for cultivations on HSG medium into account, an amount of 30 mg plasmid DNA is expected to be found in the lysate. This theoretical value is in good correspondence to experimental values determined for cleared lysates, *i.e.* 25 mg L^{-1}. A significant amount of plasmid DNA was lost due to the loss of liquid during centrifugation and filtration. In the classical procedure, 80 % of the lysate liquid were recovered and could be used for subsequent purification which corresponded to 24 mg of plasmid DNA. In case of the continuous lysis in combination with

froth flotation about 50 % of the lysate liquid covered the flotated material and could not be recovered by simple draining. Therefore, only 15 mg could theoretically be recovered.

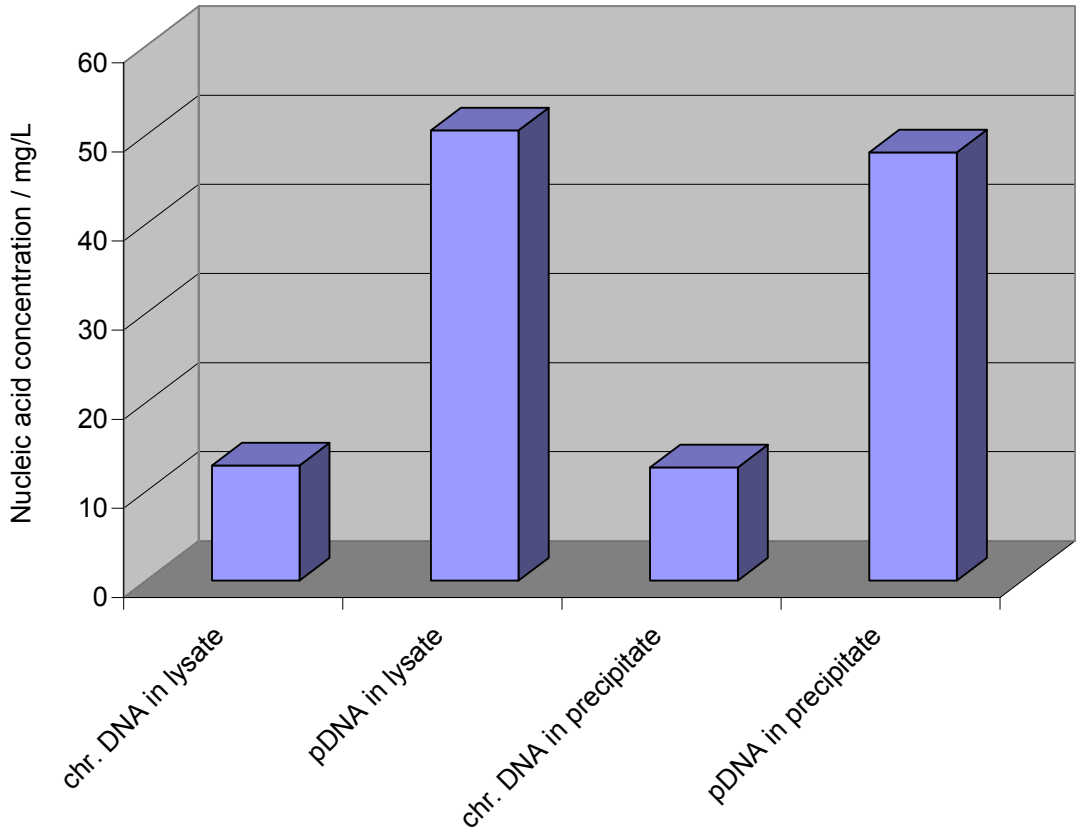


Figure 27 Plasmid and chr. DNA concentrations in the lysate liquid obtained after froth flotation and in the washing liquid obtained after rinsing the precipitate with an equal volume of buffer (Tschapalda, 2008).

However, the flotated material could be rinsed with the same volume of a suitable buffer and, thus, the plasmid containing liquid (corresponding to additional 15 mg of plasmid DNA) could be completely recovered from the flotation column. Figure 27 shows the concentrations of plasmid DNA and chr. DNA in the drained lysate liquid in comparison to the liquid obtained after rinsing the flotated material. Plasmid concentrations were comparable to the concentrations in the drained lysate. In addition, it could be shown that the concentration of chr. DNA in the volume obtained after washing did not exceed the concentration found in the drained lysate. In this way, plasmid DNA recov-

ery could be increased significantly without additional contamination by chromosomal DNA.

4.2.2 Contamination of Cleared Lysates with Genomic DNA

Genomic DNA may be susceptible to shear and result in small DNA fragments contaminating the product stream (Levy *et al.*, 2000). Therefore, the liberation of genomic DNA during different alkaline lysis protocols was analyzed and compared to the continuous operation with subsequent froth flotation. *E. coli* cells from the same batch cultivation were disrupted by continuous lysis, inverting, stirring in a beaker at 200 rpm and 600 rpm using an Intermig stirrer and stirring in a bottle using a magnetic stirrer at 400 rpm. The continuous lysis/flotation procedure and the "manual" lysis using inverting for mixing of the liquids were carried out as described in the preceding section. For the lysis procedure using the Intermig stirrer, a 5 L beaker having an inner diameter of 17 cm was used. The diameter of the stirrer was 11 cm, resulting in a ratio of 0.65. The lower impeller was located 3.3 cm above the beaker's bottom while the second impeller was located 8.5 cm above the lower impeller. The beaker was filled with 1 L of a homogeneous biomass suspension (10 g wet cell paste per litre). A volume of 1 L of lysis buffer was added and mixed for 5 minutes. Then, 1 L of neutralization buffer was added and the liquids were mixed for 5 minutes again. The content of chromosomal DNA was measured by realtime PCR amplification of a partial pyruvate kinase sequence from *E. coli*.

As presented in Figure 28 the continuous lysis procedure combined with flotation gave the lowest chromosomal DNA concentration with 14 mg L^{-1} while a high application of energy through strong mixing at 600 rpm resulted in the highest concentration of chr. DNA with 56 mg L^{-1}. Inverting as well as stirring at low frequency gave rather low values of genomic DNA in the lysate that were almost comparable to the continuous operation (both 20 mg L^{-1}). Interestingly mixing with a magnetic stirrer in a bottle resulted in a rather high concentration of chr. DNA with 46 mg L^{-1}.

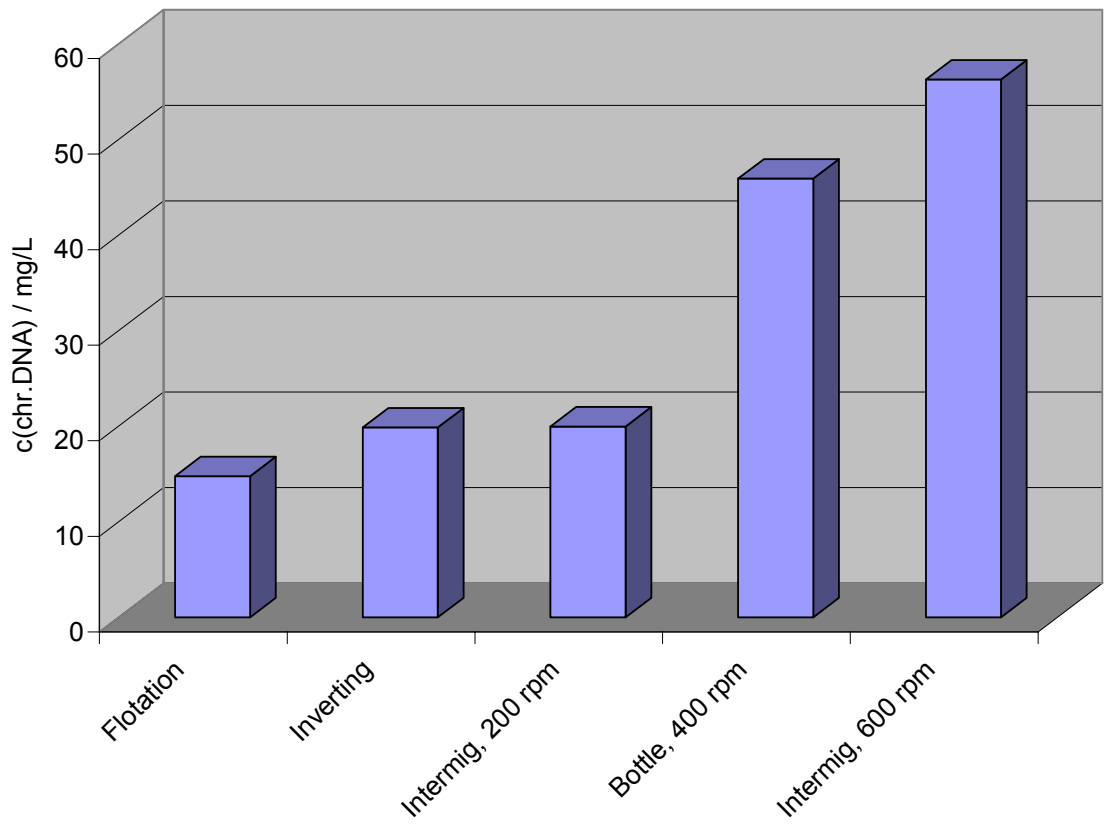


Figure 28 Comparison of chromosomal DNA concentrations found in cleared lysates obtained by different lysis procedures. The conditions are described in the text.

4.2.3 Conclusions

A semi-continuous process for large scale cell disruption by alkaline lysis was described. By the combination of a continuous lysis procedure with froth flotation for clarification, large amounts of biomass could be disrupted in a very short time. For optimum product isolation, flow rate and residence time during lysis were optimized. Mixing of the lysed cells with neutralization buffer in the column was achieved by sparged air that was used for froth flotation.

With respect to product recovery, the cell disruption processes were compared to a "manual" lysis according to common plasmid purification protocols where mixing was achieved by inverting. Incubation times for alkaline lysis and subsequent neutralization were in good aggreement with optimal values determined by Clemson and Kelly (2003).

Urthaler *et al.* (2007) described a process where the concentration of plasmid DNA in the lysate was comparable to the concentration obtained by manual isolation on a small scale. They also showed that the continuous processing had no adverse effects on plasmid form distribution. Hebel *et al.* (2006) did not mention any product recoveries at all. Furthermore, the lysate obtained had to be clarified by filtration before subsequent purification could take place. O'Mahony *et al.* (2007) applied thermolysis for plasmid isolation from *E. coli*. Optimized conditions were only capable to isolate between 35 and 54 % of the plasmid DNA that was found after alkaline lysis. It should be noted that the papers did not discuss product loss due to a decrease of cleared lysate volume after clarification. Although froth flotation resulted in rather reduced cleared lysate volumes (50 %), plasmid DNA could be sufficiently recovered by washing the flotated material.

Although the concept of continuous lysis has been described occassionally (Urthaler *et al.*, 2007; Wan *et al.*, 1998) and even a comparable process to ours has been described (Hebel *et al.*, 2006), the method presented here has not been analyzed in such details yet. The process described by Urthaler *et al.* (2007) works with specially designed equipment as does the froth flotation in our case. Although the process is well analyzed with respect to plasmid form distribution and concentration of proteins, LPS, and RNA, the concentration of the most significant contamination in this step was not determined, *i.e.* chromosomal DNA. The same applies for the work of Hebel *et al.* (2006). Here, the depletion of chromosomal DNA was only analyzed by agarose gel electrophoresis which is a rather improper analytical technique for this purpose.

Here, a special focus was set to chromosomal DNA concentration in the lysate prepared by the combination of continuous lysis and flotation. The results showed that this method was very gentle and chromosomal DNA concentrations were very low, especially in comparison to other modes of mixing.

Subsequent purification should be able to remove the different amounts of chromosomal DNA from the cleared lysate. However, it should also be noted that the efficiency of these purification protocols might not only depend on the concentration but also on the size distribution of the DNA. While the depletion of chromosomal DNA has already been described for purifications based on chromatographic procedures, the capabilities of alternative techniques, *e.g.* extraction, are not well known. For this reason, the results

obtained here will be picked up again in the chapter dealing with the extraction of nucleic acids with reverse micellar two-phase systems.

4.3 Production of a Recombinant RNase and Its Application

In comparison to state of the art procedures to remove RNA from pharmaceutical plasmid preparations, the application of RNase is still the most simple and convenient method. However, with respect to regulatory issues and safety concerns associated with the transmission of spongiforme encephalopathies, the application of RNase A from bovine pancreas is not suitable. Nevertheless, RNases are common enzymes that can also be found in microorganisms. The RNase from *Bacillus amyloliquefaciens* has occasionally been described in the literature and was considered to be an interesting alternative to RNase A. In this chapter, the production of RNase Ba in *E. coli* and its application for plasmid purification is described.

First of all, a suitable expression system had to be established in order to supply sufficient amounts of this enzyme for further research. The resulting *E. coli* clone had to be cultivated and analyzed with respect to recombinant protein expression. Finally, a purification scheme had to be established that would allow RNase Ba production in sufficient quality.

4.3.1 Cloning and Expression of a Recombinant RNase Ba

The genes of RNase Ba and its cytosolic inhibitor Barstar have been isolated from *B. amyloliquefaciens* wildtype (Bacillus Genetic Stock Centre) by PCR as described by Voss *et al.* (2006) and Lindau (2004) and integrated into a pET20b(+) expression vector (Figure 29 (A)) that allowed the periplasmic localisation of the expressed protein using the pelB export sequence. The resulting vector pET20b(+)-BarstarBarnase (Figure 29 (B)) has been transformed in *E. coli* K12JM109 (DE3) that allows to induce protein expression under the control of the T7 promoter by the addition of IPTG. The successful cloning of the genes has been verified by restriction digestion and PCR.

Plasmid pMT1002 (Figure 29 (C)) uses the Pr promoter from *E. coli* λ phage under the regulation of the cI repressor (Okorokov *et al.*, 1994). This vector was a gift from Robert Hartley who did extensive structural analyses on RNase Ba and its interaction with Barstar. In addition, pMT1002 facilitates periplamic translocation utilizing the phoA leader peptide.

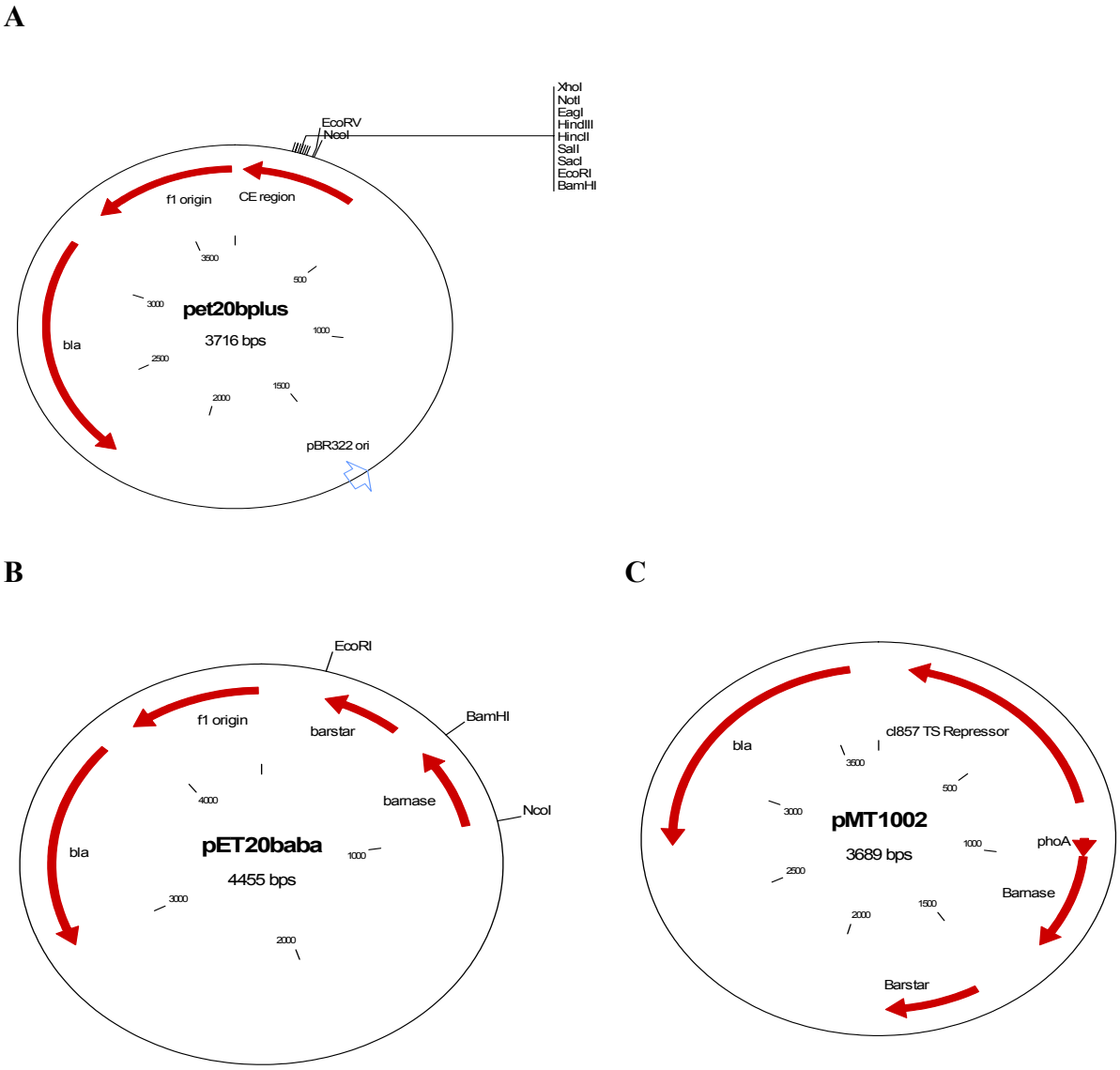


Figure 29 A: Vector pET20b(+) (Novagen) used for cloning and expression of RNase Ba. CE region: cloning and expression region, containing T7 promoter (369C – 353C), ribosomale binding site (302C – 298C), pelB leader sequence (289C – 224C), multiple cloning site (NcoI – XhoI, 225C – 158C), His tag (157C – 140C), and T7 terminator (72C – 26C). B: Vector for Barnase expression under the control of a T7 promoter. C: Expression vector for RNase Ba production used in comparison (Okorokov *et al.*, 1994).

4.3.2 Cultivation of Bacillus amyloliquefaciens on HSG Medium

In order to compare the protein expression with the RNase Ba expression in *Bacillus* as well as in a system already described in the literature, cultivations of *B. amyloliquefaciens* wild type, *E. coli* K12JM109 (DE3) pET20b(+)-BarstarBarnase, and *E. coli* XL1-blue pMT1002 have been performed in a 7 L bioreactor. For *E. coli* cultivation two media have been chosen that are commonly used at the Chair of Fermentation Engineering for microbial fermentation, *i.e.* HSG and TB medium. The composition of HSG medium has already been described in the previous chapter. TB medium contained glycerol, tryptone, and yeast extract. The complete compostion of TB medium is described in the appendices. The *Bacillus* cultivations were performed only on HSG medium and should serve as a minimum reference. All cultivations have been performed with a working volume of 5 L under the same conditions. Stirrer frequency was controlled by relative dissolved oxygen concentration in the medium. When reaching a setpoint below 60 % DO the frequency was increased by 2 % in a 30 s interval, starting with an initial value of 200 min^{-1}. The pH was kept constant at 7.0 by the automatic addition of 10 % o-phosphoric acid or 1 M sodium hydroxide solution. Temperature was kept constant at 37 °C and aeration was achieved with 5 L min^{-1}. In case of the *B. amyloliquefaciens* cultivations, average dry biomass concentrations of X = 9.7 g L^{-1} have been achieved. RNase activity was measured by an assay established by Rushizky *et al.* (1964) quantifying the concentration of soluble ribonucleotides. The parameters obtained from two cultivations are summarized in Table 17.

Table 17 Cultivation of *B. amyloliquefaciens* in a 7 L bioreactor on HSG medium as described by Lindau (2004). Yield coefficient is calculated with respect to 42 g L^{-1} media components.

Cultivation parameter	Average values
t_{cult} / h	16
X_{max} / g L^{-1}	8.5
a / kU mL^{-1}	0.58
L_v / kU L^{-1} h^{-1}	36.1
$Y_{X/S}$ / -	0.20

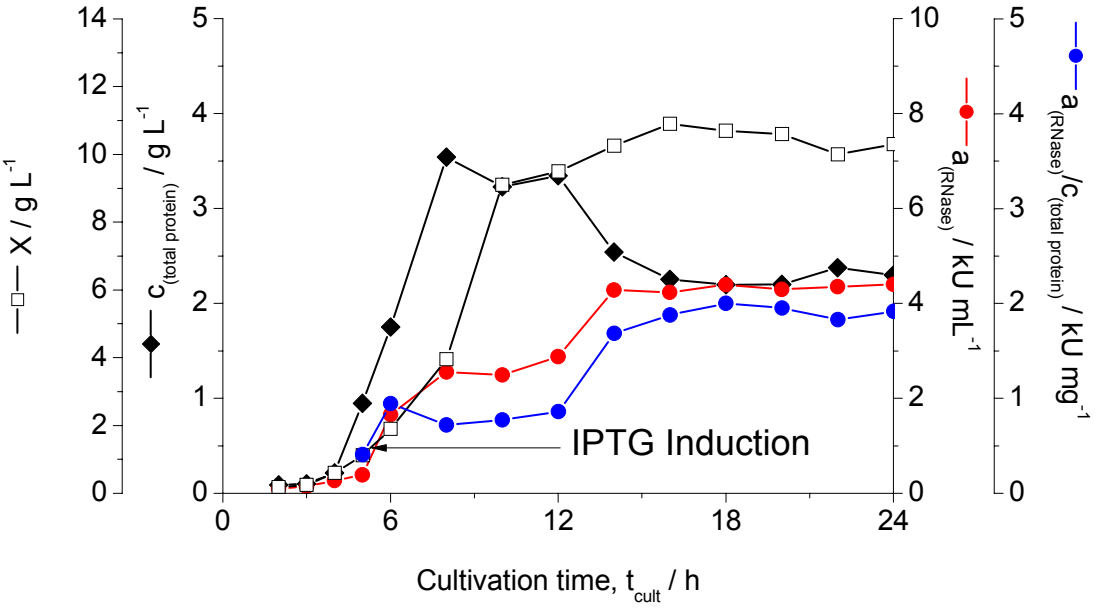
Hartley and Rogerson (Hartley and Rogerson, 1972) utilized a defined medium with sucrose as the carbon source for large scale production of RNase Ba with *B. amyloliquefaciens* wildtype which is completely different in comparison to our cultivation. In addition they did not mention the biomass concentration obtained. Therefore, a comparison with respect to productivity and yield coefficients is not possible here, but we chose to use our cultivations as a reference for future cultivations with recombinant *E. coli*.

4.3.3 Cultivation of E. coli K12 JM109 (DE3) pET20b(+)-BarnaseBarstar on Semidefined Media

Induction with IPTG to a final concentration of 10 mmol L^{-1} was accomplished after 5 hours of cultivation. Minimum RNase activity could also be observed earlier. The reason for this was considered to be a basal expression of T7 RNA polymerase in the pET expression system used or RNase originating from *E. coli*.

As shown in Figure 30 (A) for cultivations in HSG medium, a maximum RNase concentration was reached after 14 hours while total protein concentration was falling and decreased even further in the stationary phase which could be explained by lysis of dead cells during this cultivation period. At the end of the cultivation an activity around 4.4 kU mL^{-1} was achieved that remained constant over a period of 10 hours in the stationary phase. This fact was especially important because it outlined the stability of the RNase during cultivation and also gave a preferably large window for cell harvest. The productivity of this cultivation was calculated with L_V = 183.3 kU L^{-1} h^{-1} while the yield coefficients had values of $Y_{X/S}$ = 0.26, $Y_{P/S}$ = 104.5 kU g^{-1} with respect to 42 g L^{-1} of media components. The selectivity was calculated to $S_{P/X}$ =404 kU g^{-1}. These values will be compared later with culivations on TB medium and the cultivations using the pMT1002 expression vector. In case of TB medium (Figure 30 (B)) growth was faster with a shorter lag-phase during the main cultivation. RNase expression was induced after 5 hours and reached a maximum value of 3.78 kU mL^{-1} during stationary phase. Productivity, yield coefficients, and selectivity were calculated and are summarized in Table 18.

A



B

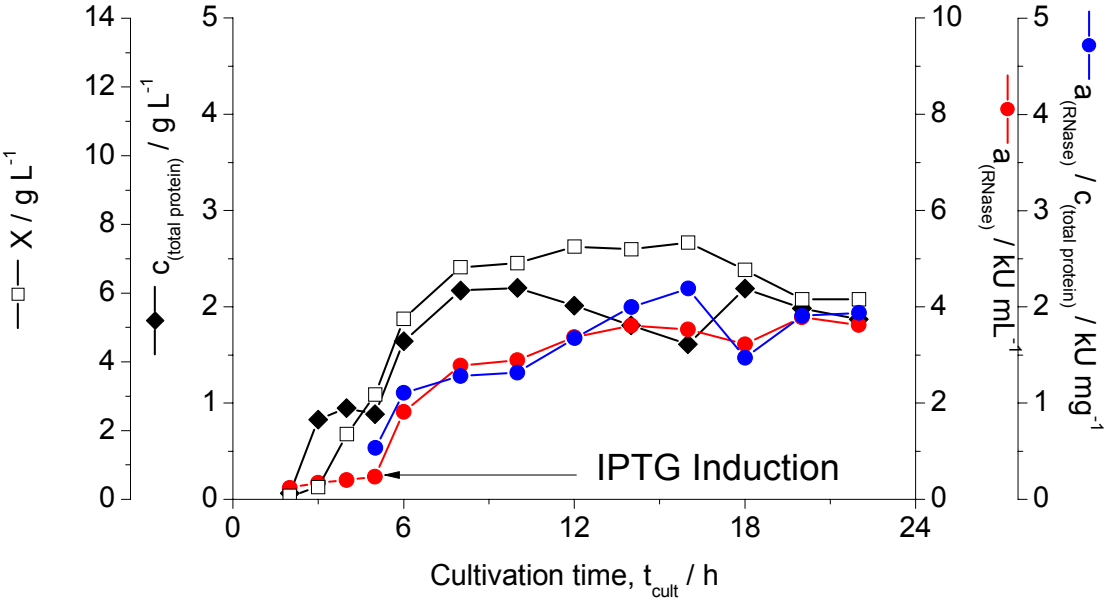
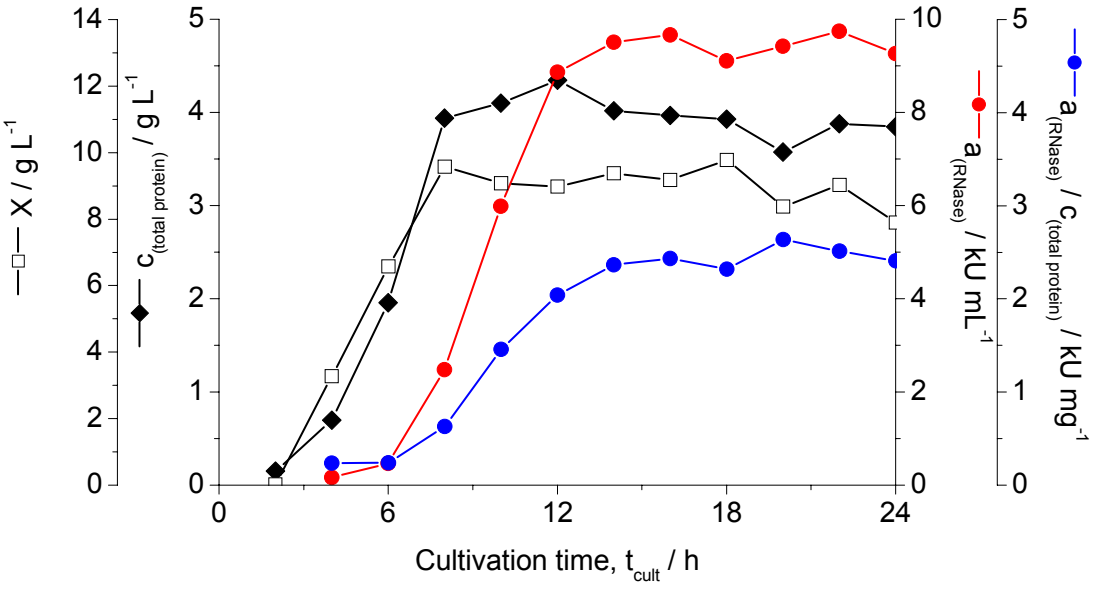


Figure 30 Growth, protein concentration, and enzyme activity for the cultivation of *E. coli* K12JM109 (DE3) pET20b(+)-BarnaseBarstar on HSG (A) and TB medium (B) in a 7 L bioreactor.

4.3.4 Cultivation of E. coli XL1-blue pMT1002 on Semidefined Media

Plasmid pMT1002 was used as a reference system for RNase Ba production. The parameters for cultivations on HSG and TB medium are presented in Figure 31.

A



B

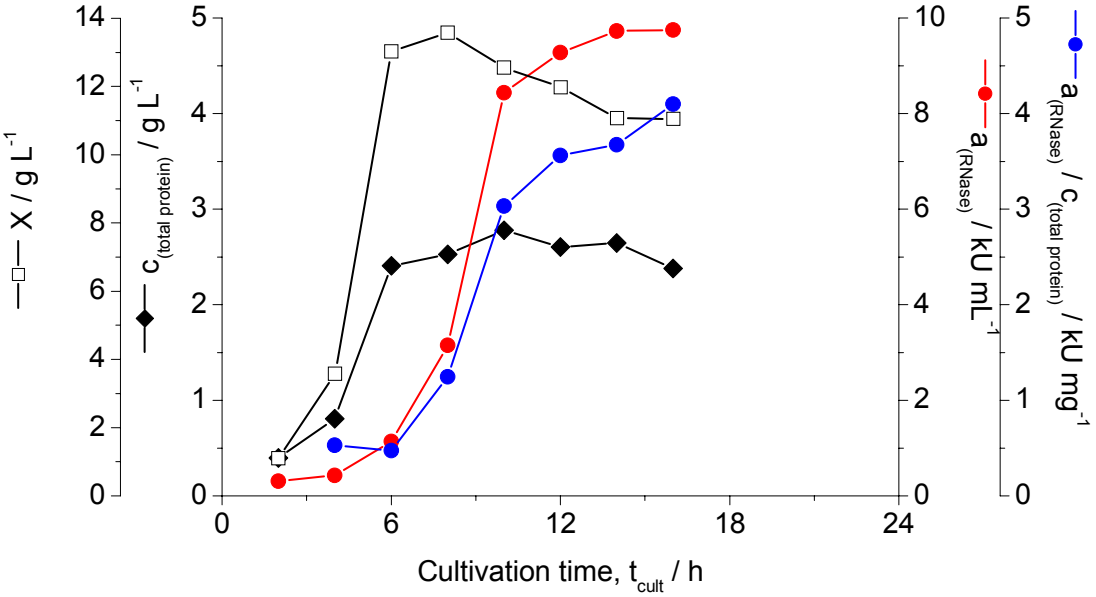


Figure 31 Growth, protein concentration, and enzyme activity for the cultivation of *E. coli* XL-1 blue pMT1002 on HSG (A) and TB medium (B) in a 7 L bioreactor.

In case of HSG medium, a comparable biomass concentration to the cultivations of *E. coli* K12JM109 (DE3) pET20b(+)-BarnaseBarstar was measured. RNase activity reached its maximum in the stationary phase and remained stable. In case of TB medium a higher biomass concentration was achieved with X = 13.6 g L^{-1}. As observed in all cultivations before, RNase activity remained at its maximum value during stationary phase. It should also be noted that *E. coli* XL-1 blue was growing much faster on TB medium and stationary phase was reached after 6 hours already.

The prolonged stability of RNase Ba was also confirmed by SDS-PAGE as shown in Figure 32.

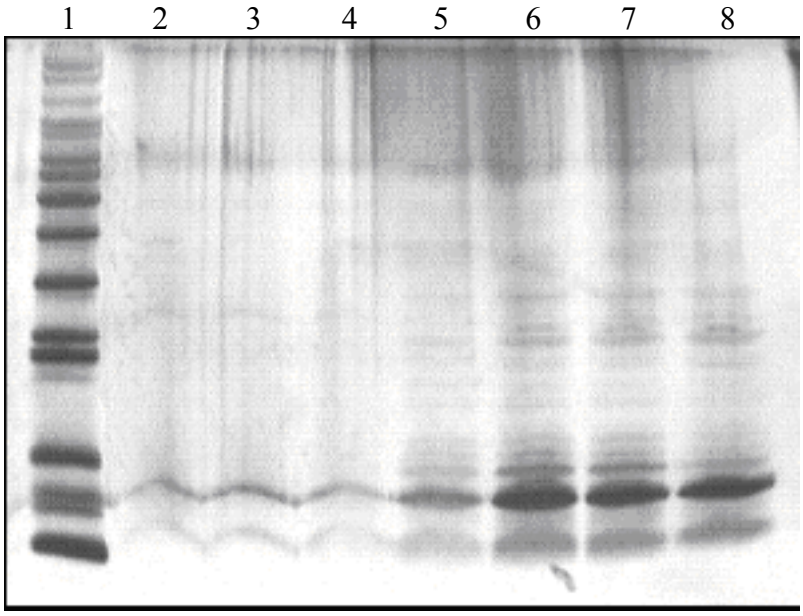


Figure 32 Analysis of the periplasmic protein fraction obtained from the cultivation of *E. coli* XL-1 blue pMT1002 on TB medium. Lane 1: marker. Lane 2 – 8: Samples taken after 4, 6, 8, 10, 12, 14, and 16 h.

The results from these cultivations are summarized in Table 18 as well. The *E. coli* XL-1 blue pMT1002 expression system showed higher activities of a = 9.74 kU mL^{-1} in comparison to the pET20b(+) system with averages values of a = 4.0 kU mL^{-1}. The reason for this could be the induction method used or the different signal peptides in both expression systems. In case of the pET system, protein expression was induced by the addition of IPTG while the pMT1002 system used a thermoresponsive repressor. Addi-

tionally, the pET system worked with pelB as a signal peptide for periplasmic protein translocation while the pMT1002 system used the phoA signal peptide.

Table 18 Comparison of the different cultivations for RNase Ba production. Yield coefficients $Y_{X/S}$ and $Y_{P/S}$ were calculated with respect to the concentration of all media components (HSG: 42 g L^{-1}; TB: 55.8 g L^{-1}).

	E. coli JM109 (DE3) pET20b(+)-BarnaseBarstar		*E. coli* XL1-blue pMT1002	
Parameter	HSG	TB	HSG	TB
t_{cult} / h	24	22	24	16
X_{max} / g L^{-1}	10.9	7.5	9.8	13.6
a / kU mL^{-1}	4.4	3.62	9.73	9.74
L_v / kU L^{-1} h^{-1}	183.3	164.6	405.4	608.8
$Y_{X/S}$ / -	0.20	0.13	0.23	0.24
$Y_{P/S}$ / kU g^{-1}	104.5	64.9	231.1	174.6
$S_{P/X}$ / kU g^{-1}	404	483	993	716

In comparison to the *Bacillus* cultivation described in Table 17 the expression with recombinant *E. coli* was more efficient due to the expression systems used. Biomass concentrations and the yield coefficient $Y_{X/S}$ were comparable to the cultivation performed with *Bacillus amyloliquefaciens* wildtype.

4.3.5 Downstream Processing of RNase Ba

Sufficient amounts of RNase Ba had to be purified to test its efficiency in RNA hydrolysis during plasmid purification. For this purpose a simple purification scheme was established. In a first capturing step, RNase Ba was isolated from the periplasm by the addition of acetic acid until a pH of 4.5 was reached. Under these conditions a lot of periplasmic proteins were also precipitated simplifying subsequent purification. The lysate obtained was clarified by centrifugation and applied to a XK 16/20 column packed with SP-Trisacryl and equilibrated with 50 mM NaOAc (pH 4.5). Elution was

obtained by a step gradient to 2 M ammonium acetate (pH 8.0). The chromatogram of the elution as well as the SDS-PAGE analysis of the product fractions are shown in Figure 33.

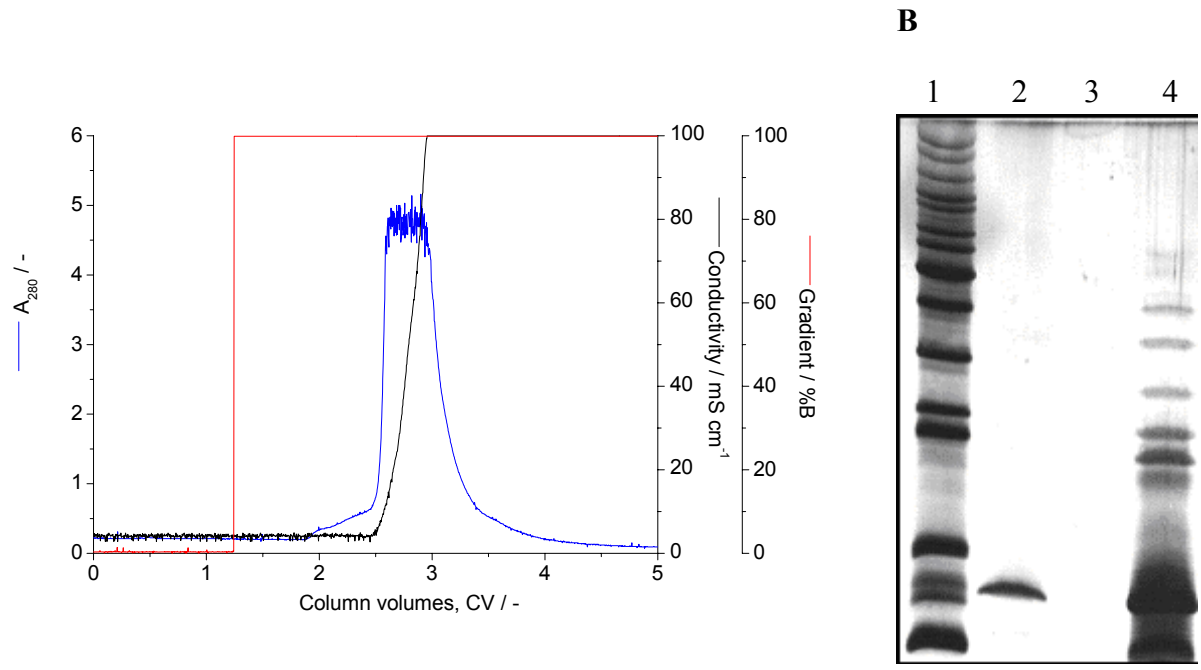


Figure 33 Capturing of RNase Ba from a cultivation of *E. coli* XL-1 blue pMT1002 on TB medium. Part A: Elution profile of cation exchange chromatography on SP-Trisacryl. Part B: SDS-PAGE analysis of fractions from cation exchange purification. Lane 1: marker. Lane 2: Applied sample. Lane 3: Flow through. Lane 4: Eluted fraction.

The concentrated fraction obtained by step elution showed a large band with a size of 12 kDa and several other proteins. The 10 kDa band could be associated to the inhibitor while the other bands constituted further impurities. A second cation exchange chromatography was used for additional polishing. Therefore, the RNase Ba fraction from the capturing step was dialyzed against 40 mM sodium acetate solution (pH 4.5) over night and applied to a XK 16/20 column packed with SP-Trisacryl. The chromatogramm and the electrophoretic analysis of the protein fractions are shown in Figure 34. The electrophoretic analysis showed that the contaminating proteins could be depleted further and RNase Ba was recovered in sufficient purity in fractions 8 and 9 while fraction 7 still contained significant amounts of the inhibitor barstar. The success of this purification

scheme is summarized in Table 19. It should be noted, that an elution with a linear gradient in a one step cation exchange purification did not lead to significant purity (data not shown). Therefore, this two step procedure with initial capturing and additional polishing was chosen.

A **B**

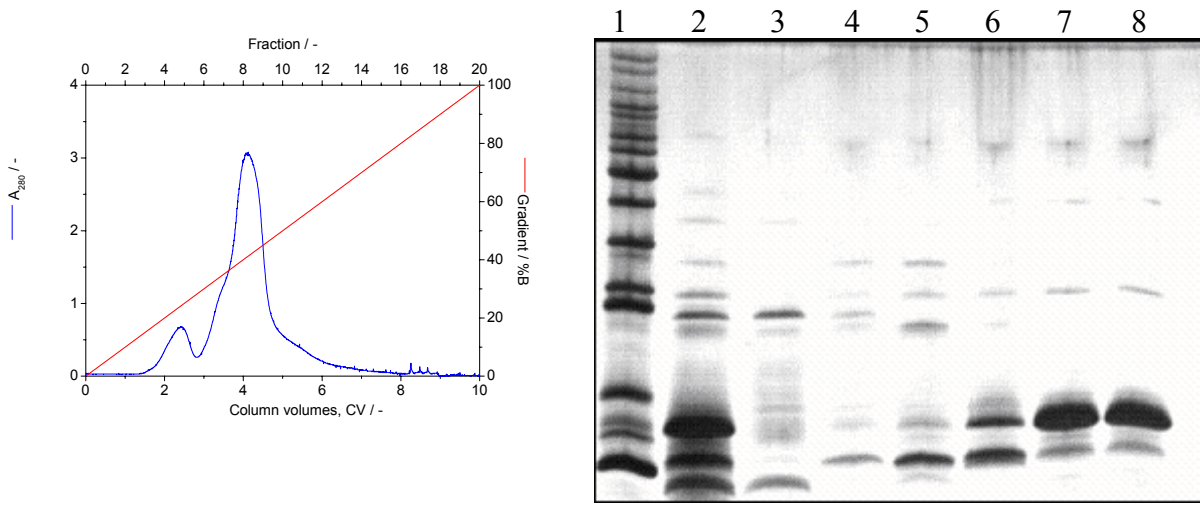


Figure 34 Second cation exchange chromatography of RNase Ba fraction captured by SP-Trisacryl. Part A: Elution profile using a linear gradient over 10 column volumes. Part B: SDS-PAGE analysis of the elution fractions. Lane 1: marker. Lane 2: loaded sample. Lane 3: flow through. Lanes 4 – 8: elution fractions 4, 5, 7, 8, and 9.

Table 19 Purification of RNase Ba using a two step chromatographic procedure.

Step	Volume	Activity	[a] Concentration factor	Total protein concentration	Specific activity	[c] Concentration factor
	mL	kU mL^{-1}	-	g L^{-1}	kU mg^{-1}	-
Culture	2000	1.05	1	0.46	2.28	1
SP-Trisacryl	25	24.64	23.5	2.65	9.3	4
Dialysis	40	14.32	13.6	2.06	6.94	3
SP-Trisacryl	20	[b] 25.6	24.38	[b] 0.95	[b] 26.95	11.8

[a] with respect to RNase Ba activity in the culture.

[b] only fractions 8 and 9 from second cation exchange step were analyzed here.

[c] with respect to the specific enzyme activity.

The results showed that a first capturing step by cation exchange chromatography was useful. A volume reduction by a factor of 80 was achieved here while specific activity was increased by a factor of 4. This indicated that contaminating proteins have been depleted in this initial step. Dialysis showed a slight decrease in overall protein concentrations and specific activity which was associated to the dilution of the sample by this method. Buffer exchange by diafiltration using a 5 kDa ultrafiltration membrane proved to be more effective in subsequent purification runs. The final polishing step allowed an additional volume reduction by a factor of 2. The RNase activity was only increased by a factor of 1.8 which confirmed a minimal loss of RNase during this step that could also be observed in the SDS PAGE analysis. Specific activity was increased by a factor of 3.9 which was explained by the removal of other contaminating proteins in this step which was indicated by a reduction of the total protein concentration by a factor of 2.2. In conclusion, enough RNase was isolated to be applied in a plasmid purification process. In case of RNase A, concentrations of 100 mg L^{-1} in the cell resupension buffer

were used. This value was considered as a reference for the concentration of RNase Ba in such experiments.

4.3.6 Application of RNase Ba for Purification of Plasmid DNA

Purified RNase Ba was used at a concentration of 100 mg L^{-1} in the cell resuspension buffer. For a first analysis of the ribonucleolytic activity, plasmid harboring *E. coli* were resuspended in this buffer and subjected to alkaline lysis according to common protocols (Birnboim and Doly, 1979; Voss *et al.*, 2006). Samples from these experiments were analyzed by agarose gel electrophoresis and compared to samples that were lysed with RNase A and without any RNase. The analysis is shown in Figure 35 (A).

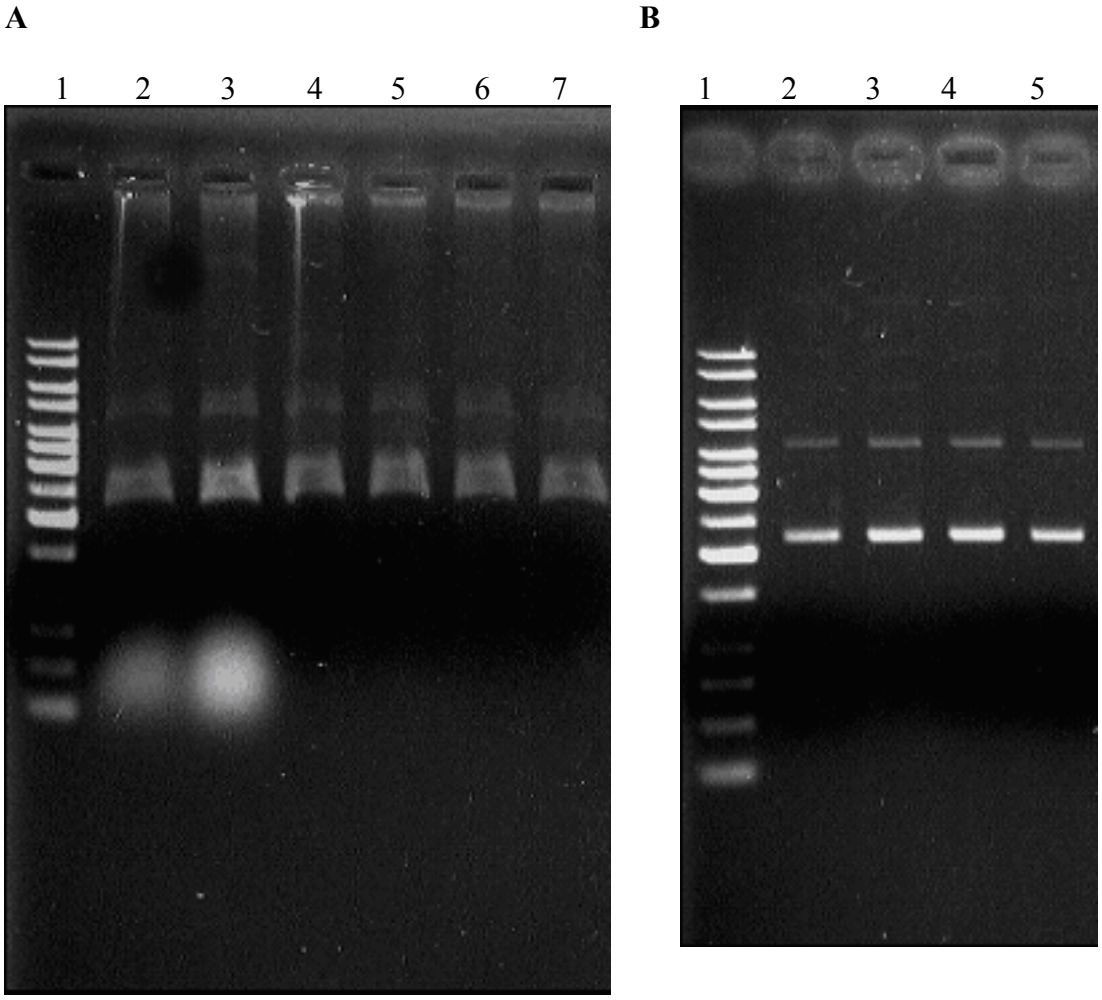


Figure 35 Agarose gel electrophoresis of cleared lysates (A) and plasmid DNA after purification with anion exchange chromatography (B). Part A: Lane 1: PEQ Lab 1kb DNA-Ladder, lane 2, 3: lysate without enzymatic removal of RNA, lane 4, 5: lysate treated with bovine RNase A (100 µg mL^{-1}), lane 6, 7: lysate treated with microbial RNase Ba (100 µg mL^{-1}). In each case 10 µL of cleared lysate were analysed. Part B: Lane 1: 1kb DNA-Ladder, lane 2, 3: plasmid DNA pUT649 obtained after isolation with RNase A, lane 4, 5: plasmid DNA pUT649 obtained after isolation with RNase Ba (10 µL of eluted fraction).

The results showed that no significant amount of RNA is visible after treatment with the enzyme. In case of RNase Ba a comparable degradation of the ribonucleic acid was observable as in the case of RNase A. When looking at the plasmid DNA, no significant DNase activity could be observed at this stage. Figure 35 (B) shows the plasmid DNA

after purification with a QIAGEN anion exchange resin. The plasmids prepared with RNase Ba showed the same form distribution as the ones prepared with RNase A. However, to analyze this subject further, purified plasmid samples have also been analyzed by capillary gel electrophoresis to detect small changes in the ccc-oc ratio. In order to exclude any DNase activity in the purified RNase Ba, a plasmid solution was incubated with barnase for a period of 1 hour at room temperature. This solution was then analyzed by capillary gel electrophoresis as already described in Voss *et al.* (2006). The plasmid sample that was used for this experiment showed a ccc monomer content of 97 % while the oc forms constituted only 3 %. After incubation with RNase Ba 96 % of the plasmid DNA were found in the ccc monomer form while 4 % were oc forms. The difference of 1 % was still within the margin of error for this experiment and it could be concluded that no DNase is active. Since a highly purified enzyme was considered to be to expensive, it was also analyzed if a crude preparation that was obtained after capturing with a cation exchanger could be used. Figure 36 shows the SDS PAGE analysis of both enzyme preparations used.

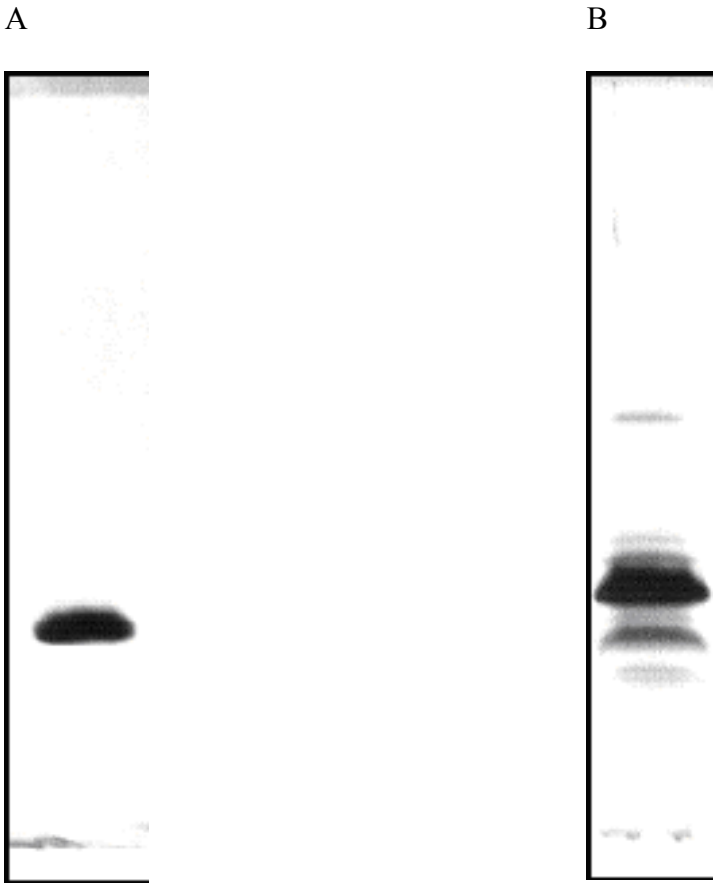


Figure 36 SDS PAGE analysis of two different RNase Ba preparations. A: homogeneous preparation. B: heterogeneous preparation.

The heterogeneous preparation was tested under the same conditions as the purified enzyme and found to be equally capable to degrade RNA in a plasmid purification process. Additionally, no DNase activity was found in this preparation by CGE analysis.

4.3.7 Conclusions

The results presented showed that RNase Ba from *Bacillus amyloliquefaciens* could be produced in sufficient quality and quantity by recombinant expression in *E. coli* and subsequent purification. The enzyme showed a comparable activity to RNase A while no deleterious DNase activity could be found. The general applicability of this enzyme however depends on its commercial availability. For this purpose the production proc-

ess has to be optimized with respect to the expression system and the purification protocol which is part of current research.

It has to be taken into account that RNase Ba does not have to be compared to RNase A when looking at its economic feasability, but to other processes like size exclusion chromatography or extraction procedures that are currently used in pharmaceutical plasmid DNA production. In comparison to chromatographic procedure, this recombinant enzyme is certainly a worthy alternative. Furthermore, the feasability can be increased by immobilization on appropriate matrices that can be reused.

4.4 Protein-DNA Interaction for Affinity Purification

Affinity separation techniques for plasmid purification have been described occasionally. Most common is the application of triple helix formation (Ito *et al.*, 1992; Schluep and Cooney, 1998). The application of DNA binding proteins for this purpose has been described recently (Woodgate *et al.*, 2002). Since DNA affinity or in other terms sequence specific recognition is a common principle in nature, it seemed only obvious to apply such an interaction for selective purification of plasmid DNA. As a first step, two DNA binding proteins have been produced by recombinant expression in *E. coli*, *i.e.* the well known Lac repressor (LacI) and the replication initiator protein RepU of the rolling circle plasmid pUB110. Both proteins were modified with a His_6 tag by cloning into an appropriate expression vector. The hexahistidine residue should serve two purposes. The first was of course a simple purification by IMAC while the second purpose was an oriented non covalent immobilization on different stationary phases (Figure 37). The building block principle also facilitated the reuse of the chromatographic matrices.

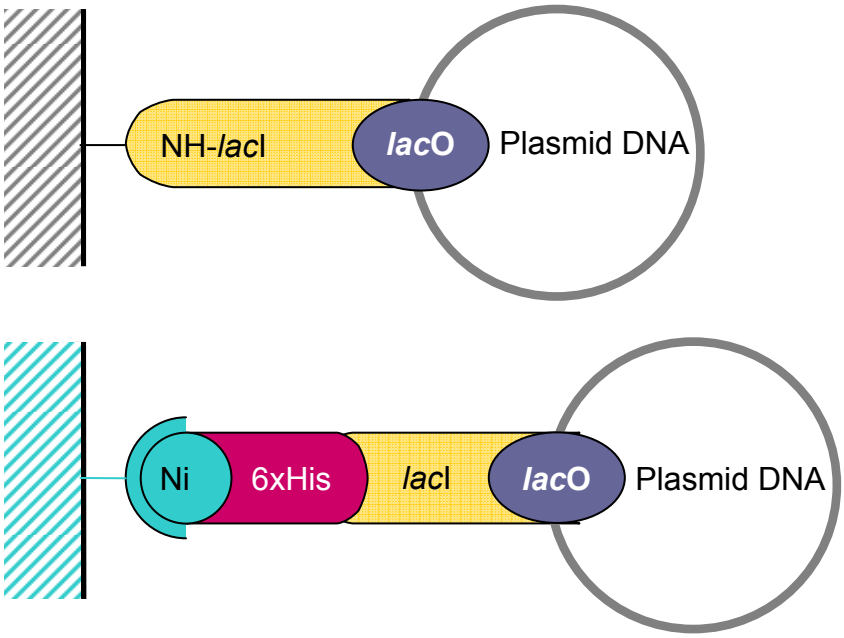


Figure 37 Principle of covalent and non-covalent immobilization (Hasche, 2004).

However, before the proteins were attached to a chromatographic matrix, their DNA binding properties had to be examined by a fast and convenient method. For this purpose, the shift in the electrophoretic mobility of nucleic acids interacting with the DNA

binding proteins was analyzed. Proteins binding to the nucleic acids would evidently decrease their specific charge resulting in a lower electrophoretic mobility of the complex (Matejtschuk, 1997). Proteins that caused this mobility shift were used for tests on chromatographic material.

4.4.1 Application of LacI for Affinity Purification

The lac repressor sequence was amplified by PCR and transformed into the vector pET20b(+) as described in Hasche (2004) and Hasche and Voß (2005). Next to the His_6 tagged protein a native repressor was purified which was used in comparison. Figure 38 compares the DNA binding activity of the native LacI to the activity of the His_6 tagged one. RNA and a PCR amplified lac operator sequences were used as binding partners for the proteins.

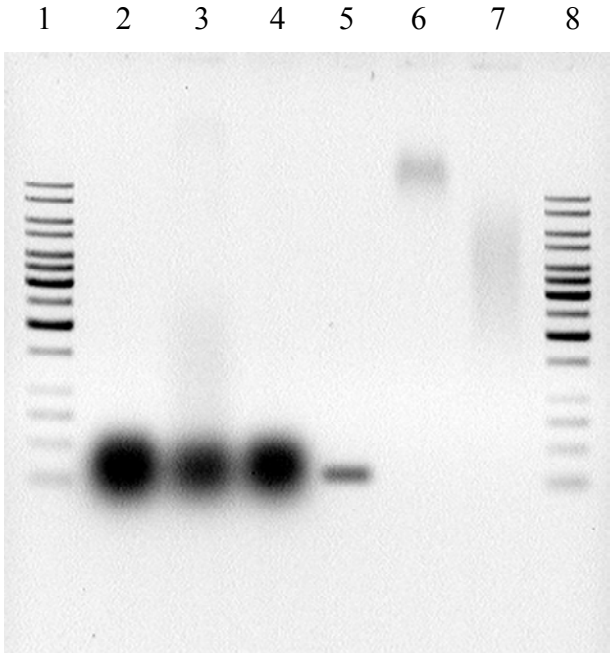


Figure 38 Binding between lac repressor proteins and nucleic acids (negative agarose gel). 1, 8: 1 kb DNA marker. 2: RNA. 3: RNA and LacI. 4: RNA and LacI-His_6. 5: Lac operator fragment. 6: Lac operator fragment and LacI. 7: Lac operator fragment and LacI-His_6 (Hasche and Voß, 2005).

The lanes 1 and 4 show the pure nucleic acids while the lanes 2 and 5 show mixtures with the native LacI and 3 and 6 show mixtures with LacI-His_6. In case of RNA, no interaction with the proteins was detectable while in the event of the lac operator sequence the nucleic acid was retarded in its migration through the agarose gel when contacted with LacI or LacI-His_6. These results showed that the proteins preferably bound to double stranded DNA and did not attach to RNA. It was indicated that the interaction of the native LacI with DNA was stronger than the interaction with LacI-His_6.

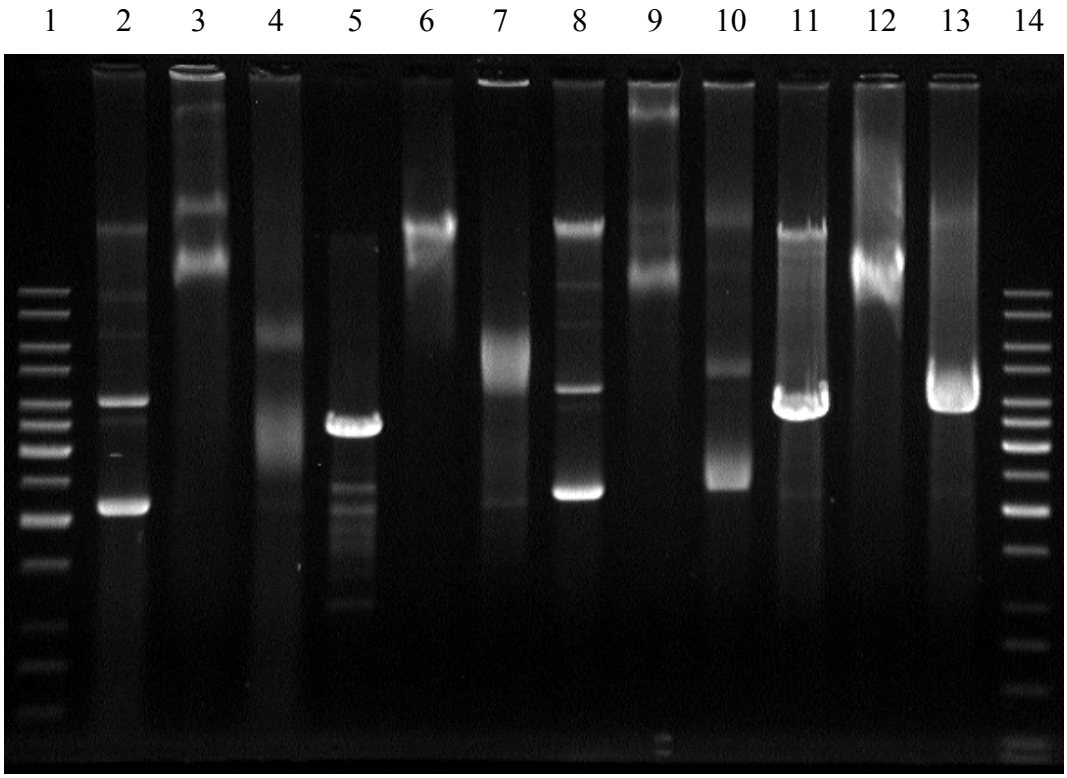


Figure 39 Binding between lac repressor proteins and plasmid DNA. 1, 14: 1 kb DNA marker. 2: pQE30. 3: pQE30 with LacI. 4: pQE30 with LacI-His_6. 5: pQE30 after EcoRI digestion. 6: pQE30 after EcoRI digestion with LacI. 7: pQE30 after EcoRI digestion with LacI-His_6. 8: pET20b(+). 9: pET20b(+) with LacI. 10: pET20b(+) with LacI-His_6. 11: pET20b(+) after EcoRI digestion. 12: pET20b(+) after EcoRI digestion with LacI. 13: pET20b(+) after EcoRI digestion with LacI-His_6. Concentration of LacI: 14.2 mg mL^{-1}. Concentration of LacI-His_6: 1.35 mg mL^{-1}. Plasmid amount per lane: 300 ng. Protein concentrations were determined by using the Bradford assay (Hasche and Voß, 2005).

Additionally it could be concluded that the binding was not simply electrostatic interaction but must have been double strand selective. The binding activity of both proteins with respect to plasmid DNA is shown in Figure 39.

Here, 300 ng plasmid DNA samples were incubated with 2 µL of the repressor proteins and the migration behavior was analyzed by agarose gel electrophoresis in comparison to the untreated DNA. Plasmid pQE30 contained a lac operator site for sequence specific binding of the repressor while pET20b(+) lacked this genetic element. However, in both cases the electrophoretic mobility is shifted indicating an interaction of the repressor proteins with the DNA. Since interaction occurs with undigested and linearized DNA it could be concluded that it was independent of the plasmid topology. The assay also showed that the interaction was not sequence specific but rather based on an electrostatic nature with a specifity for double stranded DNA because both plasmids were retarded equally. A difference in the binding activity between the native LacI and the his tagged repressor could not be determined because the proteins were used at different concentrations for this assay. Because LacI-His_6 showed DNA binding properties, the protein was attached to a metal chelate membrane adsorber (Sartorius) charged with Cu^{2+}. For this purpose, excess protein was loaded on the IMAC membrane and washed with IMAC lysis buffer. Subsequently plasmid DNA (pQE30) was loaded onto the modified membrane. Again, the system was washed and eluted with 10 mM IPTG solution as described in Hasche and Voß (2005). The DNA containing fractions were analyzed by agarose gel electrophoresis as shown in Figure 40.

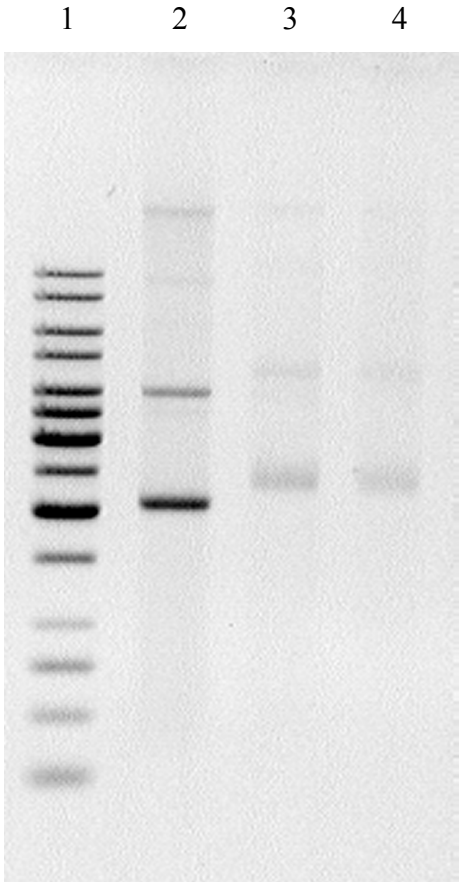


Figure 40 Binding of plasmid DNA with LacI-His_6 after non-covalent immobilization on a metal chelate membrane adsorber (negative agarose gel). 1: 1 kb DNA marker. 2: Loaded plasmid DNA sample. 3: Flow through. 4: Elution with 10 mM IPTG (Hasche and Voß, 2005).

The agarose gel shows that equal amounts of plasmid DNA were found in flow through and in the eluted fraction. However, the amount of bound material was considerably low in comparison to other methods. Because of the low expression yields of LacI-His_6 and the rather tedious purification of the native protein it was decided to focus the research on the replication initiator protein RepU which has been tested in parallel to LacI.

4.4.2 Application of RepU_F for Affinity Purification

The rolling circle replication initiator protein RepU was chosen as an affinity ligand for DNA protein interaction because intensive research was done in the field of rolling circle replication and the mechanism of replication as well as the structural elements of the

Rep proteins are already well known. In case of RepU, conserved sequences of the Rep protein family had still to be identified. This was done by sequence alignment with standard software (BioEdit) as described by Kortmann (2005).

Structural Elements of RepU

First of all, the DNA binding site of RepU had to be identified. In case of RepC, this motive was found at the C terminus of the protein at positions 263 to 279 as shown in Table 20. From the conserved arrangement of structural motives in all Rep proteins (Ilyina and Koonin, 1992) it could be concluded that the DNA binding site in RepU will also be located at the C terminal end.

Table 20 Conserved amino acid sequence of RepU and RepC (Kortmann, 2005).

Protein	amino acid sequence[b]	amino acid position
RepU	GLHRKRLISYGGLLKEI	273-298
RepC[a]	KLHRN**SRT**KYKNLIKEI	263-279

[a] essential amino acids for origin recognition are in bold letters
[b] same or comparable amino acids are marked in yellow

A comparable sequence was found in RepU at positions 273 to 298 (Table 20). The histidine at position 275 is highly conserved in several Rep protein families. Next to the DNA binding site three other conserved motives were present in the repU gene (Ilyina and Koonin, 1992). Motive 3 holds the catalytic centre. Glu-244 functions as the active amino acid and terminates replication by hydrolysis of the phosphodiester bond in the synthesized strand. In this way RepU acts like a nuclease. The second active amino acid Tyr-248 initiates the single strand break and a 5'-phosphotyrosin bond is formed between RepU and the DNA as well as a free 3'-OH end. This reaction is typical for topoisomerase I A enzymes.

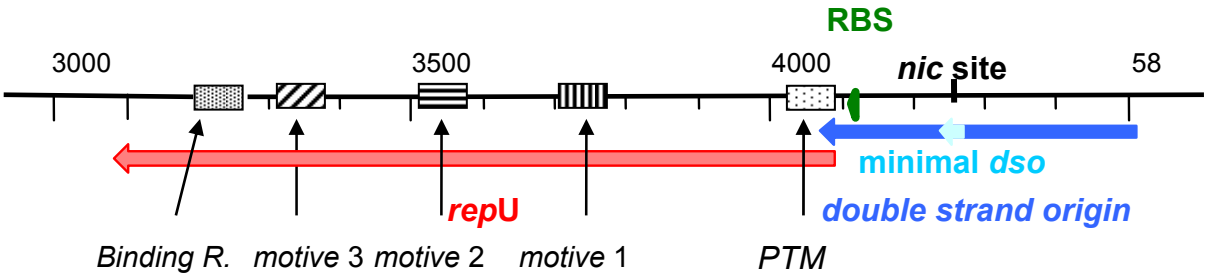


Figure 41 Schematic presentation of pUB110 region from 3000 to 58 bp showing the repU gene with three conserved motives, posttranslational modification (PTM) und DNA binding region. Additionally the ribosomal binding site (RBS), the double strand origin (dso), minimal dso and the nic site have been charted (Kortmann, 2005).

Motive 2 contained a metal binding domain while the function of motive 1 is still unknown.

The genetic sequence of repU (Figure 41) could be translated theoretically into an amino acid sequence which would represent the complete protein which is presented schematically in Figure 42.

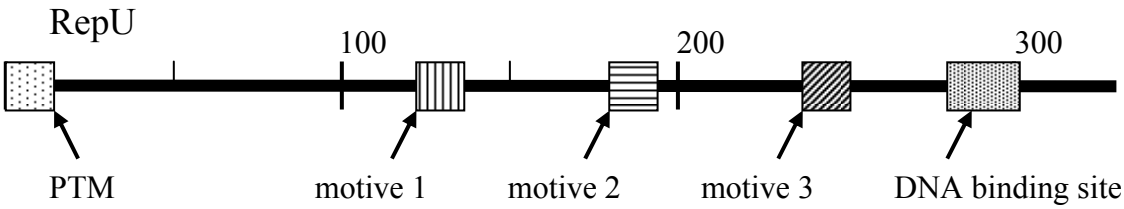


Figure 42 Schematic presentation of RepU from plasmid pUB110 showing the three conserved motives, the posttranslational modification (PTM) and the DNA binding region (Kortmann, 2005).

Based on these results different recombinant expression vectors were designed that should facilitate the expression of the complete RepU protein and smaller fragments that contained a limited number of the described motives but at least the DNA binding domain (Figure 43).

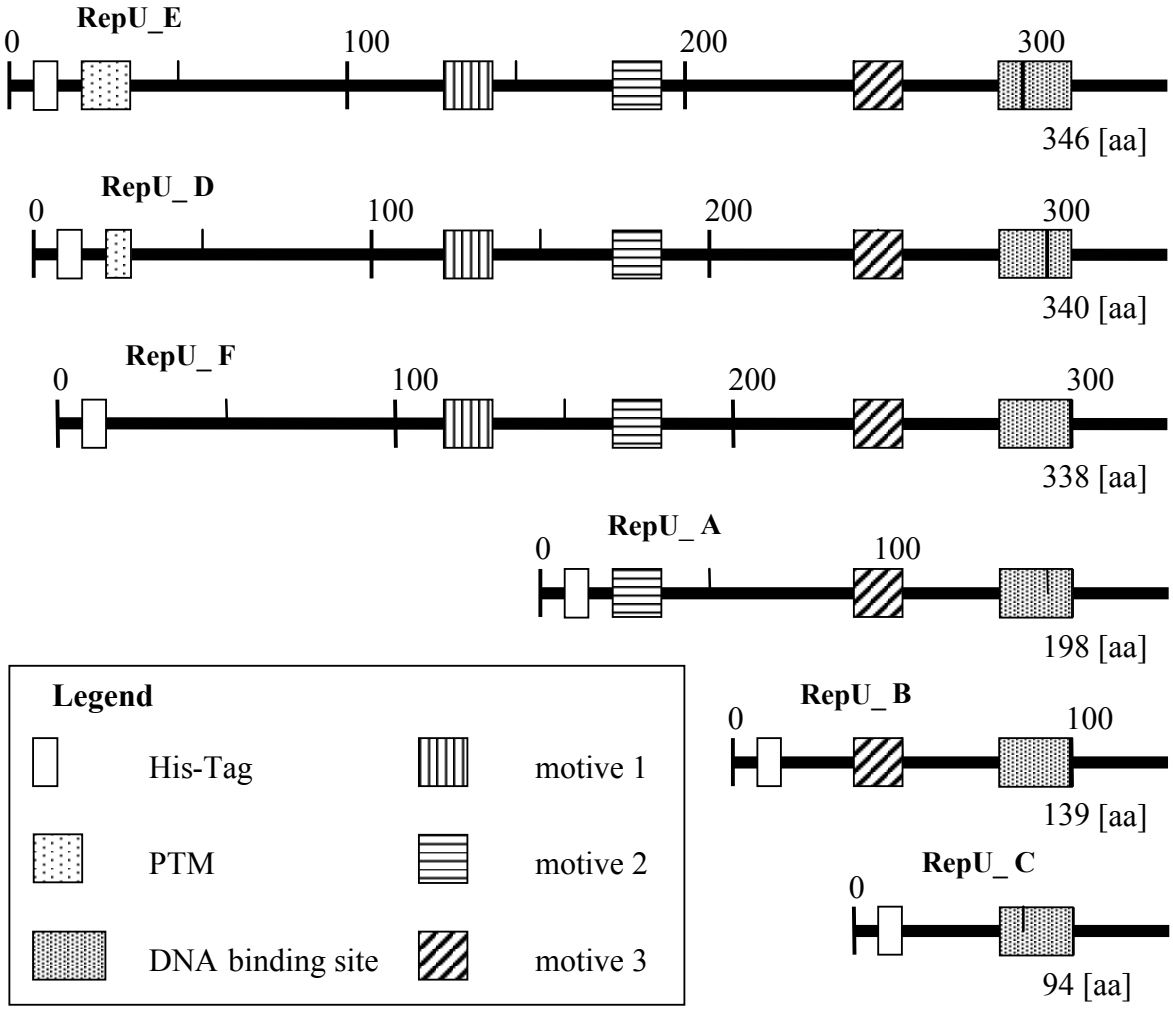


Figure 43 Schematic presentation of the different RepU fragments expressed in pQE30 (Kortmann, 2005).

As shown by western blot analysis in Figure 44 the proteins RepU_B and RepU_F could be expressed successfully. In case of the other proteins, no expressing clones could be identified. The reason for this could be a low proteolytic stability of the smaller proteins like RepU_C or the formation of inclusion bodies in all other cases.

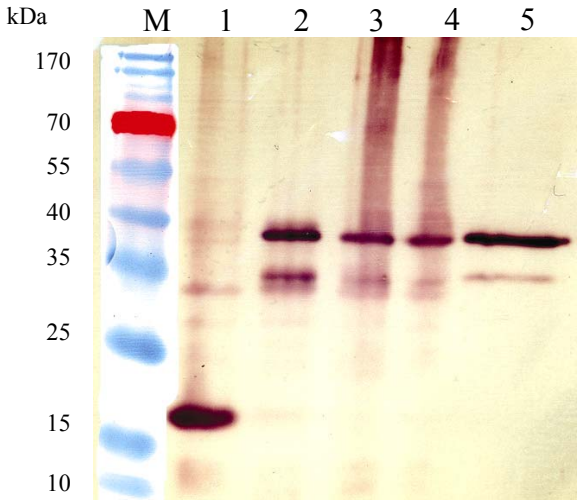


Figure 44 Western blot analysis of different culture samples after cell lysis following analytical SDS PAGE. M: Protein size marker. 1: Sample from *E. coli* M15 pREP4 pQE30_B. 2: Sample from *E. coli* JM109 (DE3) pQE30_F. 3 and 4: Samples from *E. coli* M15 pREP4 pQE30_F. 5: Elution fraction 1 from IMAC purification (Kortmann, 2005).

Purification and Activity of RepU_F

Although RepU_B could be expressed in *E. coli* the expression rate and recovery after purification was not high. Therefore, experiments were performed with RepU_F only. Purification of RepU_F could be achieved by IMAC on a Ni-Sepharose 6 FF column following standard protocols. Elution with a step gradient as shown in Figure 45 gave a fairly pure 40 kDa protein that was identified by SDS PAGE analysis presented in Figure 46. The DNA binding properties of the eluted protein were tested by the gel shift assay that was already described earlier for LacI.

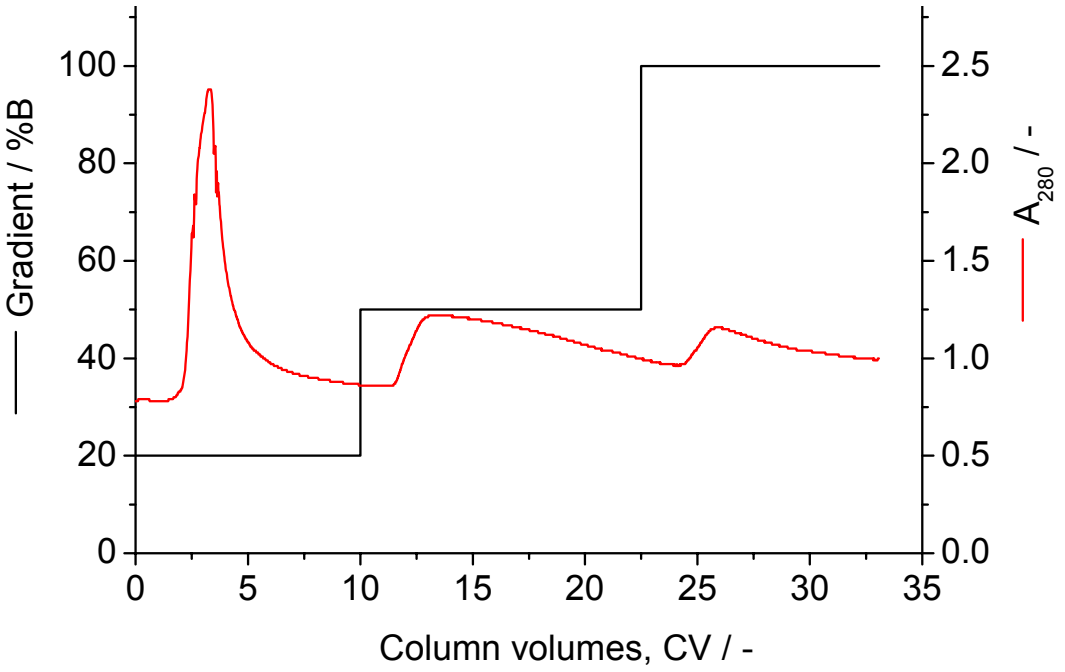


Figure 45 Elution profile of IMAC purification of RepU_F on Ni-Sepharose FF using a step gradient. Composition of buffer B: 50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole (Kortmann, 2005).

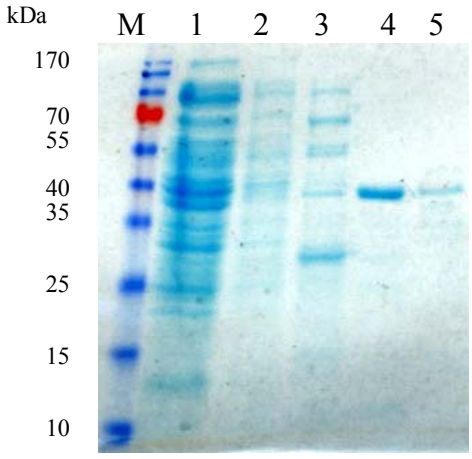


Figure 46 SDS PAGE analysis of the fractions obtained from IMAC purification of RepU_F shown in Figure 45. Lane M: Protein size marker. Lane 1: Lysate. Lane 2: Flow through. Lane 3: Elution with 20 % buffer B. Lane 4: Elution with 50 % buffer B. Lane 5: Elution with 100 % buffer B. RepU_F can be identified as a 40 kDa band (Kortmann, 2005).

Both a PCR amplified fragment of the double strand origin (dsoX) as well as a plasmid containing this genetic element (pGEM dsoX) were used as binding partners for the protein. The agarose gel is shown in Figure 47. In case of the PCR fragment a complete retardation was observed when the DNA is incubated with higher amounts of RepU_F.

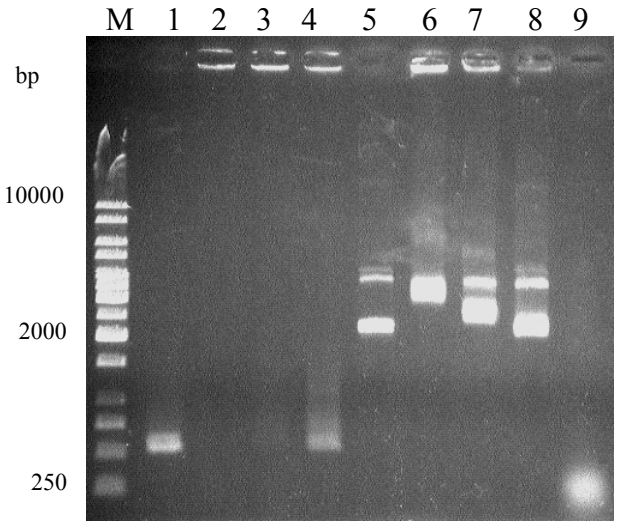


Figure 47 Gel shift analysis of the unspecific binding behavior of the isolated RepU_F fractions. As DNA targets a dsoX fragment and plasmid pGEM dsoX were used. Lane M: 1 kb ladder. Lane 1: pure dsoX fragment. Lane 2: dsoX fragment incubated with 2 µL of eluted protein. Lane 3: dsoX fragment incubated with 1 µL of eluted protein. Lane 4: dsoX incubated with 0.5 µL of eluted protein. Lane 5: pure pGEM dsoX. Lane 6: pGEM dsoX incubated with 2 µL of eluted protein. Lane 7: pGEM dsoX incubated with 1 µL of eluted protein. Lane 8: pGEM dsoX incubated with 0.5 µL of eluted protein. Lane 9: RNA incubated with 2 µL of eluted protein (Kortmann, 2005).

When the amount of RepU_F was sufficiently reduced a rather diffuse band was observed (lane 4) indicating different degrees of unspecific binding to the target DNA. A comparable observation could be made for the plasmid DNA. In comparison to the pure DNA, mixtures of the protein with the plasmid showed a shift in the electrophoretic migration. Because of the difference in size the plasmid was not completely retarded but the effect was proportional to the protein amount in the mixture being typical for non specific binding.

Non Covalent Immobilization of His_6-RepU

To avoid steric hindrance of protein DNA interaction during column chromatography a CIM disk was chosen as matrix for non covalent immobilization of the RepU_F through its His tag. The high porosity of this matrix as well as its flow properties made it well suited for this purpose. The prepurified protein was loaded onto the CIM disk. Breakthrough curves have been used to determine the dynamic binding capacity for RepU_F. After washing, the same procedure was repeated with plasmid DNA (pGEM dsoX). In this way, the dynamic binding capacity of the affinity system for plasmids could be determined as well. First experiments gave rather low binding capacities for both protein (4.4 mg mL^{-1}) and plasmid DNA (205 µg mL^{-1}) (Abel, 2006). Nevertheless, in comparison to affinity purification with a 64 amino acid lac repressor peptide immobilized on a streamline matrix that resulted in a dynamic binding capacity of 143 µg mL^{-1} (Forde *et al.*, 2006) the values described by Abel (2006) were comparably high. However, it should be noted that the matrices used for immobilization were completely different. In case of the porous particulate streamline material binding of the DNA was only expected on the outer surface of the material. In case of the CIM IDA disk binding was assumed in the complete pore surface of the macroporous monolithic disk. For this reason higher capacities could be expected with monolithic columns. The rather low dynamic capacity for proteins and thus for plasmid DNA could also be explained by the tendency of RepU_F for aggregation. This instability proved to be a serious hazard for establishing an affinity purification system. Variation in the buffer composition used for storage of the protein led to an increased stability and the breakthrough experiments could be repeated (Bellwied, 2008). It should be noted that the screening of suitable buffer conditions to enhance protein stability in solution was a rather tedious procedure and scientific output was low. The dynamic capacities are summarized and compared to already published values in Table 21.

Table 21 Dynamic binding capacities of chromatographic materials modified with DNA binding proteins.

Sample	q / mg mL^{-1}	mg_{pDNA} $mg^{-1}_{protein}$	Matrix	Reference
RepU_F	4.4	0.047	CIM IDA disk	Abel, 2006
pGEM dsoX	0.205		CIM IDA disk	
$pUC19_{lacO3/lacOs}$	0.143	n/a	Streamline	Forde *et al.*, 2006
RepU_F	23	0.017	CIM IDA disk	Bellwied, 2008
pGEM dsoX	0.4		CIM IDA disk	

The protein binding capacity of the CIM IDA disk with 23 mg mL^{-1} was in good agreement with results published by Peterka *et al.* (2006) who found a dynamic binding capacity of 18-30 mg mL^{-1} for His tagged GFP. The recovery of the plasmid DNA was complete in case of the CIM IDA disk (Bellwied, 2008) and only around 25 % with the LacI peptide on streamline adsorbent (Forde *et al.*, 2006). Taking this high recovery and the dynamic binding capacity into account, the results obtained from the RepU_F immobilization on a monolithic carrier was considered to be superior to the smaller peptide ligand. Comparing the results of Abel and Bellwied leads to the conclusion that a high ligand density was not a prerequisite for an increased binding capacity of plasmid DNA since the earlier work showed a ligand specific protein amount of 0.047 while the value was reasonably lower at higher protein load (0.017).

The most crucial property of an affinity matrix is its selectivity. Although binding capacities have been found to be high, the applicability of the matrix depends on its capability to separate plasmid DNA from other contaminants, especially RNA. For this reason the CIM IDA disk carrying RepU_F was loaded with a pGEM dsoX-RNA mixture in the presence of 0.5 M NaCl. Elution was accomplished with a linear gradient to 1 M

NaCl over 125 column volumes. As can be seen in Figure 48, a separation of plasmid DNA and RNA could be observed in principle. The chromatographic separation showed two peaks in the elution profile (data not shown). The first being significantly larger than the second with marginal baseline separation. Lane 4 shows the elution fraction of the first peak which contains only RNA while lanes 6 and 7 represent the elution fractions of the second peak showing only plasmid DNA.

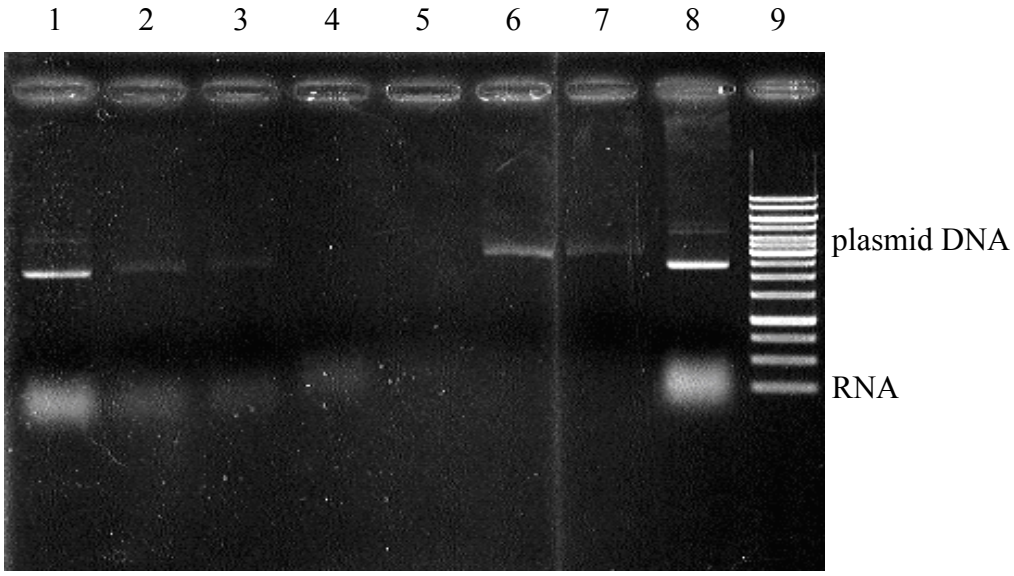


Figure 48 Agarose gel electrophoresis of the fraction obtained from a chromatographic separation of a pGEM dsoX/RNA mixture using the RepU affinity disk. Lane 1 and 8: pGEM dsoX/RNA mixture. Lane 2 and 3: Flow through. Lane 4: elution peak #1. Lane 5: peak overlap. Lane 6: elution peak #2. Lane 7: tailing of peak #2. Lane 9: 1 kb DNA marker (Bellwied, 2008).

The same experiments were repeated with plasmid pUT649 that did not contain the recognition sequence of RepU. The analysis of the chromatographic fractions is shown in Figure 49. The elution profile (data not shown) contained two peaks, but no complete baseline separation was obtained in this case. Lane 4 shows the fraction of peak #1 comprising RNA while lane 5 shows only plasmid DNA from peak #2. It can be concluded that plasmid DNA lacking the recognition sequence of RepU was not retarded equally strong as plasmid pGEM dsoX.

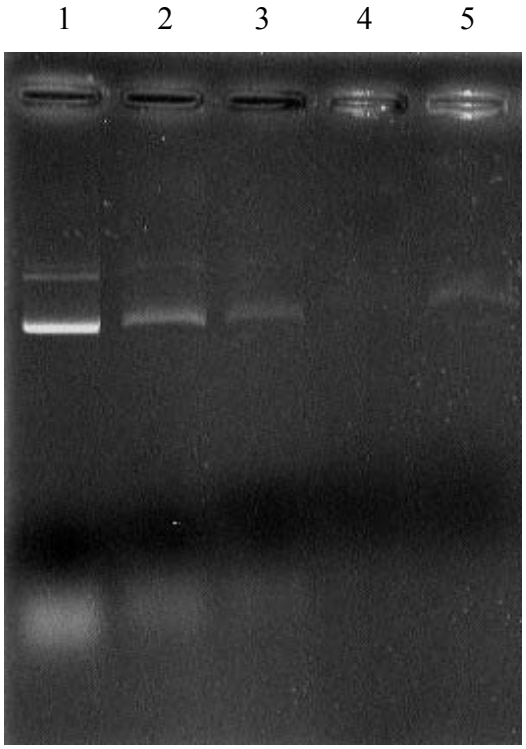


Figure 49 Agarose gel electrophoresis of the fractions obtained from a chromatographic separation of a pUT649/RNA mixture using the RepU affinity disk. Lane 1: pUT649/RNA mixture. Lanes 2 and 3: Flow through. Lane 4: Elution peak #1. Lane 5: Elution peak #2 (Bellwied, 2008).

4.4.3 Conclusions

The results described showed that a DNA binding protein could be immobilized on a chromatographic matrix and bind significant amounts of DNA. The dynamic binding capacities were comparable to the results obtained by other researchers in this field. However, the selectivity of the system did not meet the expectations. The aim of the affinity system should be to selectively bind double stranded DNA, preferably the plasmid DNA containing the sequence recognized by the DNA binding proteins. Although a DNA binding property could be demonstrated by simple gel shift assays, it could not be verified for the immobilized protein.

This low selectivity is the most serious disadvantage of these systems in the chromatographic mode. Although the sequence specifity of the applied proteins is well known

and their ability to discriminate at least between RNA and DNA was shown in gel shift assays, they were not able to completely separate plasmid DNA from RNA. DNA binding proteins usually bind to double stranded DNA in a non sequence specific manner. After this first step, the protein moves to its recognition sequence by one dimensional diffusion. The first unspecific binding is mainly based on electrostatic interaction while the sequence specific binding forms a more stable complex by addional interactions. With respect to the chromatographic process it can be assumed that the kinetics for the formation of the sequence specific complex are probably to slow and the electrostatic interaction is not capable to completely discriminate between DNA and RNA. Additionally, the instability of the protein ligands in solution and in an immobilized state makes research in this field tedious and time consuming although the stability of the RepU could be increased by screening of appropriate buffer conditions.

Due to the poor selectivity, these systems have to be examined further. At the current stage, other systems are more selective and should be preferred. This is also underlined by the structural and (bio)chemical instability of the protein ligands. Further research has also to be done in minimizing ligand size and increasing structural and chemical stability. However, the general affinity principle should also be taken into consideration and new chemically synthesized ligands should be developed and tested for plasmid DNA purification.

4.5 Extraction of Nucleic Acids with Reverse Micellar Two-Phase Systems

Extraction systems were also considered for the purification of pharmaceutical grade plasmid DNA. As already outlined, aqueous two-phase systems have successfully been applied for the removal of several impurities. Other methods based on reverse micellar two-phase systems have not yet been used for plasmid purification. Therefore, we have analyzed the potential of these systems for the production of plasmid DNA. The original intention was to use them as an initial purification step in order to remove the major impurities, especially RNA, and to concentrate the product, thus simplifying subsequent downstream processing. The following major issues had to be addressed in our research:

- How can the partitioning of the nucleic acids be influenced?
- How will surface active compounds from the bacterial cleared lysate like SDS and proteins influence the partitioning behaviour?
- Can such influences be circumvented or even be avoided?
- Is a complete separation of plasmid DNA and RNA possible?
- Can other contaminants be removed or at least be depleted as well?
- How good is the recovery?
- How can such an extraction process be integrated into a multistep purification scheme?

For the separation of plasmid DNA from RNA in water-oil-surfactant systems two general approaches could be considered as illustrated in Figure 50. It could be anticipated that the bacterial cleared lysate had to be preconditioned by diafiltration to remove surface active compounds like SDS or proteins. In principle, the lysate can be concentrated at this stage to reduce the processing volume before the first extraction step. In one process option, the RNA should be removed from the diafiltered lysate and the resulting aqueous raffinate phase should be processed further by suitable purification techniques like anion exchange chromatography. In this case, the RNA would be efficiently removed. However, the product stream would not be concentrated and might still be contaminated by other impurities like proteins, endotoxins, and chromosomal DNA. In the

second option plasmid DNA would be extracted along with RNA from the aqueous phase and in a second back extraction step only the plasmid DNA would be recovered in a new aqueous recipient phase which could be purified further by subsequent down-stream processing. With the latter process option, the plasmid DNA could be concentrated by varying the phase ratio in both extraction steps. Furthermore, both steps should allow the depletion of the other impurities found in the cleared lysate.

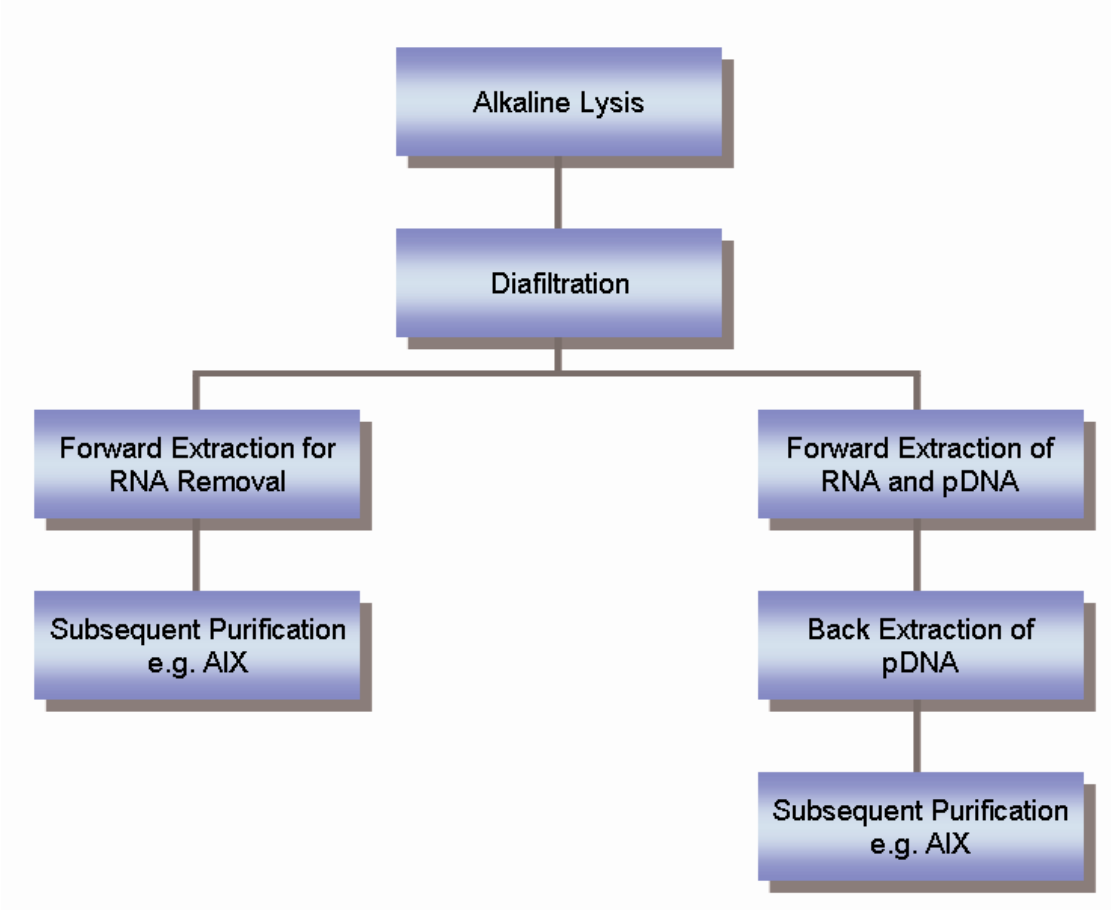


Figure 50 Process options for the purification of plasmid DNA by reverse micellar extraction systems.

Taking into account that reverse micellar extraction is mainly affected by the ionic strength of the aqueous phases the following distribution behaviour could be assumed. At low salt concentrations the electrostatic interaction between the surfactant and the nucleic acids will be strong and the reverse micelles will be comparably large resulting

in the extraction of nucleic acids into the organic phase. At moderate salt concentrations the electrostatic interaction will be weaker and the reverse micelles will be smaller. In this way only smaller molecules like RNA will be extracted from the aqueous phase while larger ones like plasmid DNA remain there. The salt concentration at which the partitioning behaviour was inverted was called the inversion point. In case of a hydrophobic masking of the nucleic acids, the cationic surfactant interacts with the negative charges on the nucleic acid backbone and in this way shields their charges by making it more hydrophobic. In this way, the nucleic acids can be dissolved in the organic phase. Separation is mainly based on the difference in hydrophobicity in these systems.

Initial studies dealt with the forward extraction process. First results were obtained with model systems and applied to bacterial lysates. As a second step, the back extraction was analyzed and optimized with respect to the depletion of contaminations originating from a lysate. Finally, the extraction process was integrated into convenient and highly scalable purification schemes and characterized with respect to the quality requirements set for pharmaceutical plasmid DNA.

4.5.1 Forward Extraction

First studies dealt with the parameters that influenced the partitioning of nucleic acids in a forward extraction step. For this purpose plasmid DNA and RNA were purified from *E. coli* and used as model systems. Plasmid DNA was separated from RNA by size exclusion chromatography and both nucleic acids were further purified and concentrated by anion exchange chromatography using Fractogel DEAE as described in Streitner *et al.* (2007). The ionic strength or salt concentration respectively was considered to be the main influencing parameter for nucleic acid partitioning in this step. When analyzing the partitioning of plasmid DNA or RNA as the only solute, the concentration of the nucleic acid has been determined by absorption measurement at 254 nm in the aqueous phases. The concentrations in the reverse micellar phase could also be determined by this method. As described later, complete partitioning of the nucleic acids into one phase was observed in most of the systems analyzed. When no complete partitioning was observed, losses occured due to precipitation at the interphase. The partitioning of plasmid DNA and RNA out of a mixture of both molecules used as model system was determined by agarose gel electrophoresis. This method was chosen to document the

separation of plasmid DNA from RNA by the analyzed extraction systems because the separation was most efficient and even small amounts of nucleic acid could be detected by ethidium bromide staining. Furthermore, a large number of samples could be processed in parallel in short time. The detection limit of nucleic acids with ethidium bromide staining is around 2 ng per lane depending on the conditions used during staining. When no nucleic acid could be detected, a partitioning coefficient of $K > 100$ or $K < 0.01$ could be assumed when a concentration of 200 ng nucleic acid per lane is applied. However, because of the complete partitioning observed, these values could converge to ∞ or 0 respectively. For this reason, it was decided not to calculate the partitioning coefficients but the residual nucleic acid content of the aqueous phase.

Salt concentration

At first, the effect of salt concentration and type was tested. Purified plasmid DNA or RNA in 10 mM Tris buffer (pH 8.0) was diluted with different salt solutions in the same buffer. The final concentration of plasmid DNA was about 25 mg L^{-1} while RNA concentration was significantly higher with 250 mg L^{-1} which reflected the nucleic acid concentration and the plasmid DNA-RNA ratio that was found in bacterial cleared lysates. The nucleic acids were extracted by isooctane containing 40 mM TOMAC and 0.5 % (v/v) ethylhexanol as a cosurfactant. The alcohol was added to enhance the solubility of the surfactant. Both phases were contacted for 2 hours at room temperature on a rotating agitator at 100 rpm. In earlier experiments it was found that 2 hours of mixing were more than sufficient for complete partitioning of the nucleic acids. Ongoing work showed that efficient mixing for just a few minutes also resulted in complete partitioning. Therefore, a period of 15 minutes was considered to be adequate. Subsequently, phase separation was achieved by centrifugation at 5000 rpm for 5 minutes.

Figure 51 shows the residual content of plasmid DNA and RNA in the aqueous phase after extraction in the presence of different alkali metal and alkaline earth metal chlorides. The nucleic acid concentration was determined by measuring the absorbance at 260 nm using a microplate reader. The general tendency that was assumed for the partitioning has been confirmed in our study. In addition, different inversion points have been determined for the alkali and alkaline earth metal chorides both for plasmid DNA and RNA. In case of $MgCl_2$ and $CaCl_2$ the inversion occured at chloride concentrations

between 140 and 160 mM for both plasmid DNA and RNA. In case of the alkali metal chlorides it occured first for LiCl at concentrations of 240 mM, than at 280 mM for NaCl, followed by 300 mM for KCl for plasmid pUT649 as presented in Figure 51 (A). For RNA the inversion occured at 280 mM LiCl, 300 mM NaCl, and 320 mM KCl. It should also be noted that $CaCl_2$ and $MgCl_2$ tended to precipitate nucleic acids which is also observed during the extraction of RNA resulting in a lower residual content as shown in Figure 51 (B).

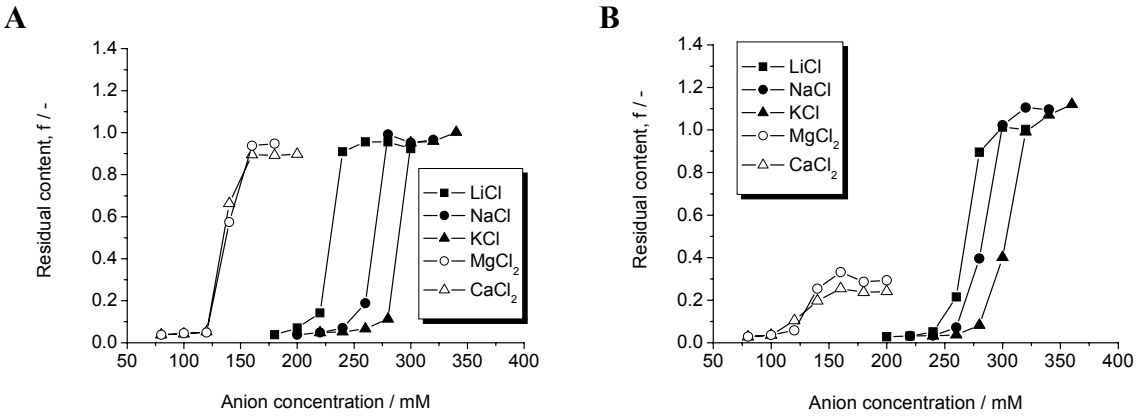


Figure 51 Residual content of plasmid pUT649 (A) and *E. coli* RNA (B) in the aqueous phase after extraction with 40 mM TOMAC in isooctane under the influence of different metal chlorides.

The results also showed that there was a difference in the inversion of the partitioning behaviour between pUT649 and RNA. In case of the alkaline earth metal chlorides this difference was rather small with an anion concentration below 10 mM, but larger in the case of the alkali metal chlorides with 40 mM for LiCl and 20 mM for NaCl and KCl. This difference was considered as a potential operation window for selective partitioning of RNA and plasmid DNA.

Furthermore, acetates, bromides, citrates, nitrates, and sulfates were tested with respect to their influence on the partitioning behaviour. In all cases the same tendencies were observed that have already been described for the chlorides. The difference in the inversion points between plasmid DNA and RNA was always largest in case of the lithium salts. Figure 52 shows the effect of different lithium salts in varying concentrations on the distribution of plasmid DNA and RNA. Inversion occured first with nitrates and

bromides followed by chlorides, sulfates, citrates, and acetates. Based on these results it was assumed that a mixture of plasmid DNA and RNA could be separated by using either acetate, citrate, chloride, or sulfate in the extraction system.

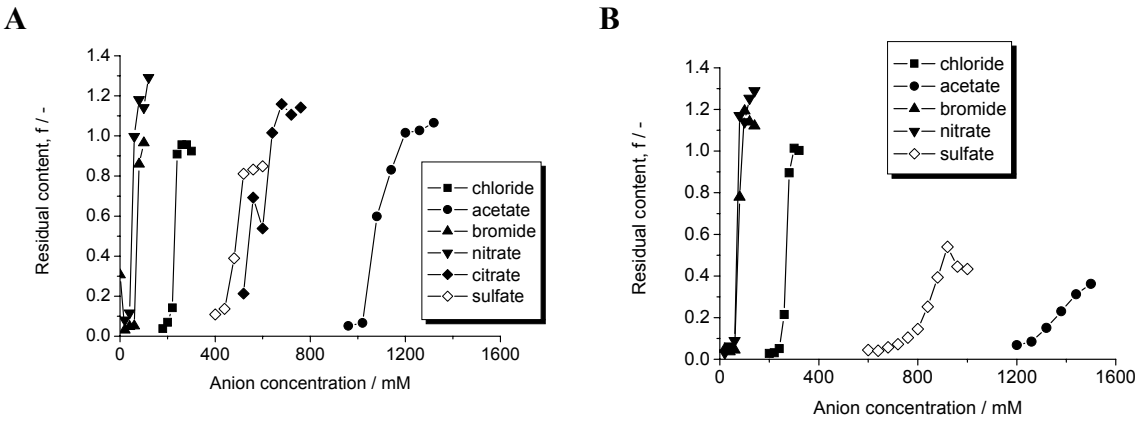


Figure 52 Residual nucleic acid content in the aqueous phase after extraction under the influence of different lithium salts for pUT649 (A) and *E. coli* RNA (B).

For this purpose, purified plasmid DNA and RNA were mixed with lithium salt solutions to achieve concentrations of 25 mg L^{-1} pUT649 and 500 mg L^{-1} RNA. The RNA concentration was increased in comparison to the initial studies in order to assure that it would remain visible during AGE analysis of the aqueous raffinate phases. The results obtained when using LiCl and LiOAc are shown in Figure 53.

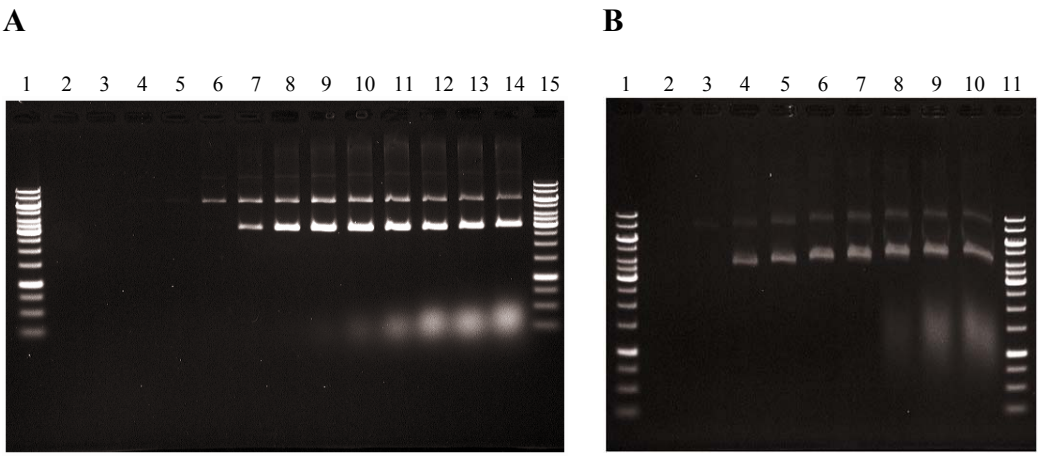


Figure 53 Agarose gel electrophoresis of a pUT649/RNA mixture in the aqueous raffinate phase after extraction with 40 mM TOMAC in isooctane in the presence of LiCl (A) and LiOAc (B). Part A: lanes 1, 15: DNA 1 kb marker; lanes 2-14: 190-310 mM LiCl in 10 mM steps. Part B: lanes 1, 11: DNA 1 kb marker, lanes 2-10: 1020-1500 mM LiOAc in 60 mM steps.

With LiCl up to concentrations of 210 mM both RNA and plasmid DNA were extracted while at concentrations between 240 and 260 mM LiCl only RNA was extracted from the aqueous phase. It should also be noted that between 220 and 230 mM LiCl both RNA and the supercoiled ccc form of the plasmid were extracted and the open circular (oc) form remained in the aqueous raffinate. A further increase of the salt concentration above 260 mM LiCl kept all nucleic acids in the aqueous phase as shown in lanes 10-14 of Figure 53 (A). In case of LiOAc the difference in the inversion point between plasmid DNA and RNA was larger in comparison to LiCl. All nucleic acids were extracted out of the aqueous phase at concentrations up to 1080 mM LiOAc (lanes 2 and 3 in Figure 53 (B)), while at concentrations between 1140 and 1320 mM only the RNA was removed and plasmid DNA remained in the aqueous phase. Again, a further increase in LiOAc concentration kept all nucleic acids in the raffinate.

Influence of Alcohol on Nucleic Acid Partitioning

Next to the ionic strength, concentration and type of the alcohol that was added as a solubilizer was also considered to influence the partitioning behaviour. In case of a reverse micellar extraction mechanism, the alcohol changes the curvature of the reverse micelles and in this way influences their structure and thus the partitioning behaviour of

the nucleic acids. In case of hydrophobic masking, the alcohol will at least modify the hydrophobicity of the solvents and in this way have an effect on nucleic acid partitioning. Under these considerations, the alcohol concentration and type used during forward extraction might not only influence partitioning in this step, but also during subsequent back extraction. In this way, separation efficiency as well as product recovery might be affected at this stage already.

With respect to the forward extraction it was found that a minimum alcohol concentration was necessary for complete dissolution of TOMAC in isooctane and in this way also for the transfer of nucleic acids into the organic phase as given in Table 22. Further, it was noted that the alcohol type and concentration changed the inversion point of the extraction systems. Figure 54 shows the change of inversion point in a forward extraction using Li_2SO_4 as salt and ethylhexanol as a solubilizer. At an alcohol concentration of 0.5 % (v/v) the inversion point was found between lithium sulfate concentrations of 520 and 560 mM. With a concentration of 1 % (v/v) ethylhexanol it was located between 360 and 400 mM, while at even increased concentration (5 % (v/v)) the inversion of the partitioning behaviour shifted to a point around 80 mM Li_2SO_4.

Table 22 Minimum amount of different alcohols relative to the organic phase volume that is necessary for a complete extraction of nucleic acids into the organic phase (Streitner *et al.*, 2008).

Alcohol	Minimum alcohol fraction / % (v/v)
Ethanol	0.04
1-Propanol	0.045
2-Propanol	0.04
1-Butanol	0.04
Isobutanol	0.05
1-Pentanol	0.05
1-Hexanol	0.06
Cyclohexanol	0.2
Ethylhexanol	0.05
1-Heptanol	0.04
2-Octanol	0.06
1-Nonanol	0.06
1-Decanol	0.07

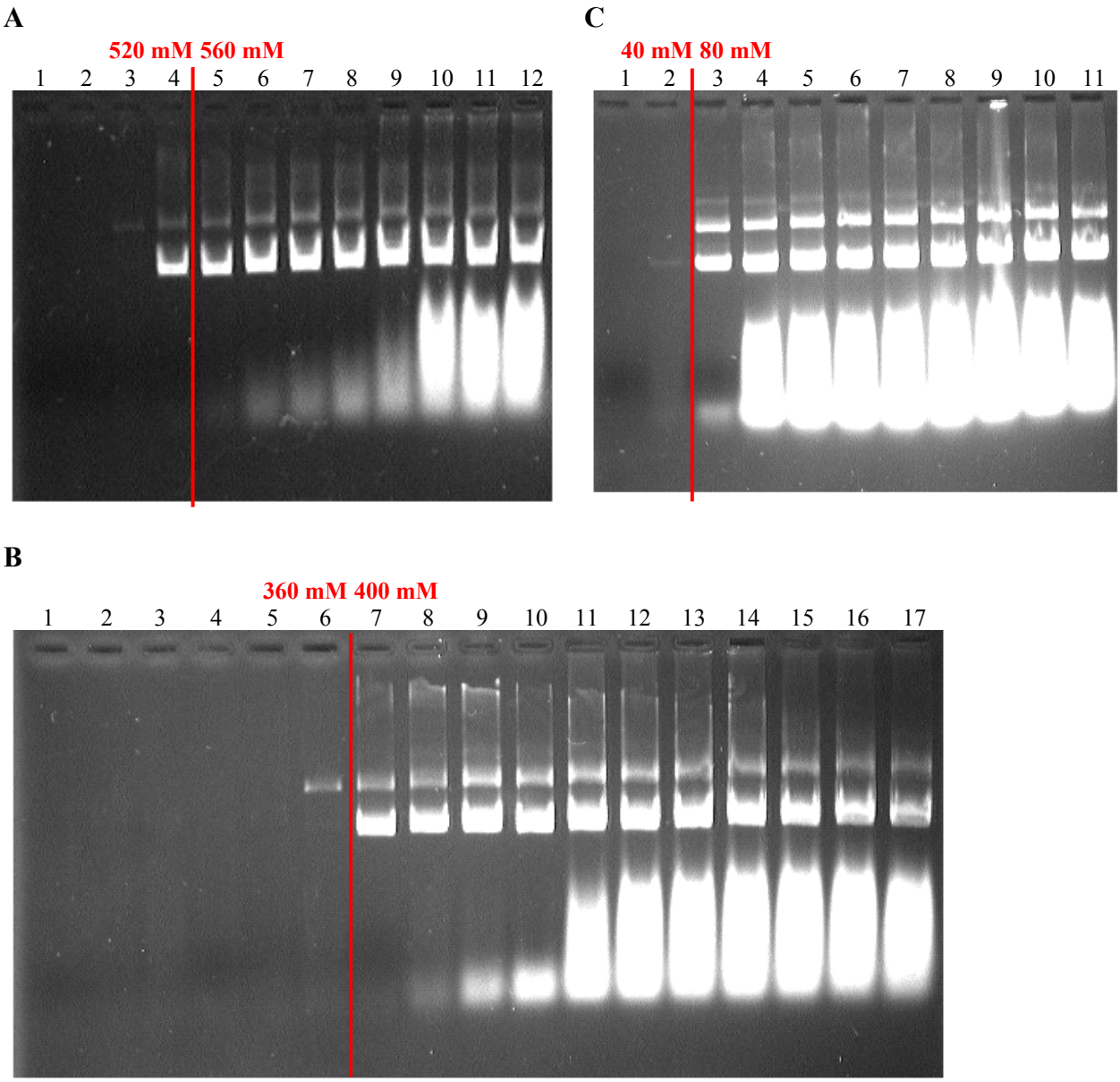


Figure 54 Change of the inversion point for extractions with Li_2SO_4 using different amounts of alcohol. A: 0.5 % (v/v) ethylhexanol. B: 1.0 % (v/v) ethylhexanol. C: 5.0 % (v/v) ethylhexanol (Streitner *et al.*, 2008). AGE analyses of the aqueous raffinate phases after extraction with a reverse micellar phase. Li_2SO_4 concentrations changed in steps of 40 mM.

Extraction from Bacterial Cleared Lysate

As already outlined in Section 4.5, it was anticipated that a separation of plasmid DNA and RNA using a bacterial cleared lysate as feed would not be successful because of the presence of SDS and proteins in this solution. The high acetate concentration was not

considered to be really problematic because our initial experiments showed that the inversion of partitioning occured at acetate concentrations above 1000 mM as shown in Figure 52.

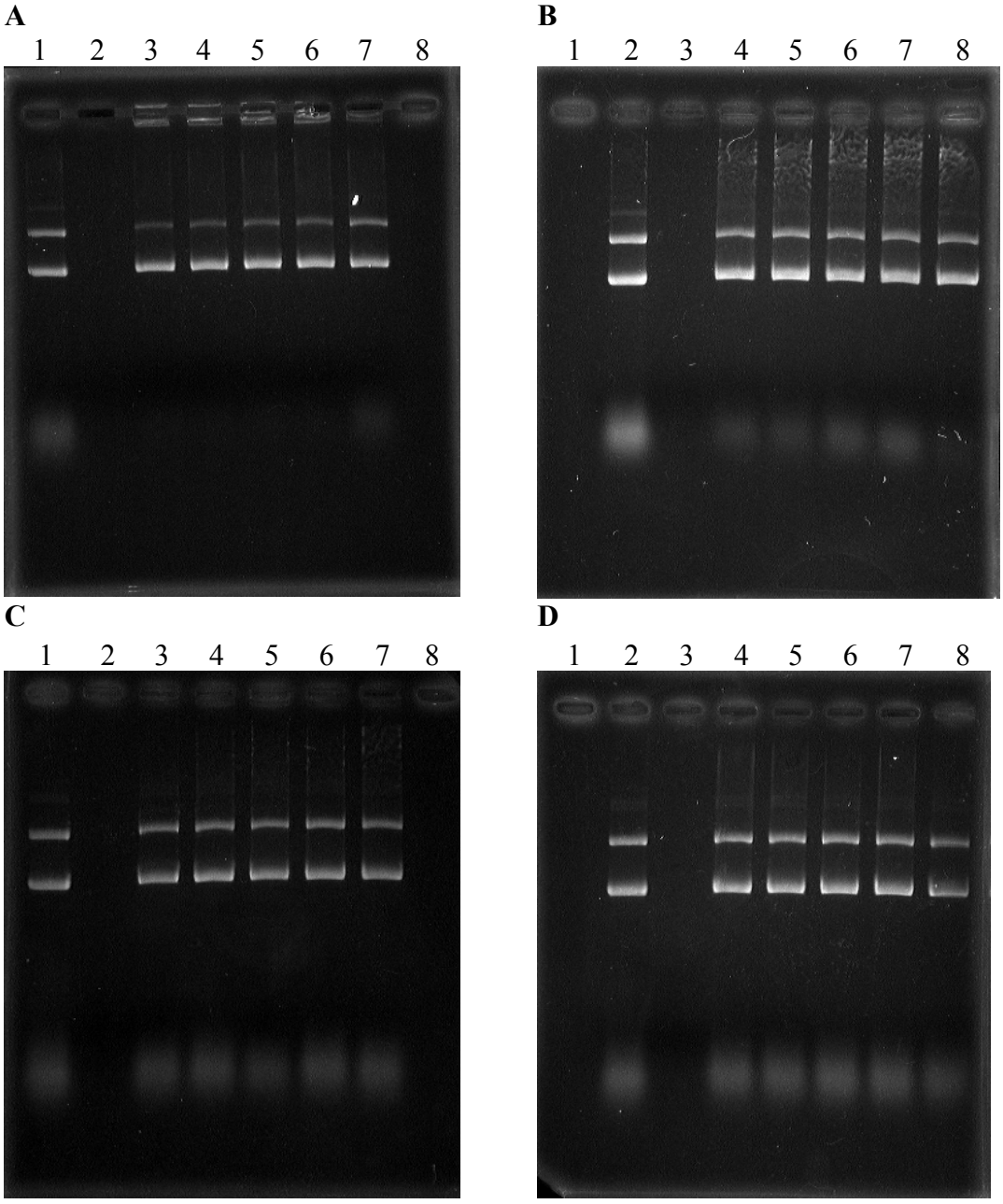


Figure 55 Effect of SDS on the separation of plasmid DNA and RNA. Extraction conditions are described in the text. A: Extraction with no SDS present. Lane 1: Aqueous feed. Lane 2: Aqueous raffinate. Lanes 3-6: Aqueous extract. B: Extraction with 0.1 % SDS. Lane 1: Aqueous feed. Lane 2: Aqueous raffinate. Lane 3-6: Aqueous extract. C: Extraction with 0.25 % SDS. Lane 1: Aqueous feed. Lane 2: Aqueous raffinate. Lane 3-6: Aqueous extract. D: Extraction with 0.5 % SDS. Lane 1: Aqueous feed. Lane 2: Aqueous raffinate. Lane 3-6: Aqueous extract.

Therefore, other compounds influenced the partitioning of the nucleic acids because a forward extraction was possible with model systems in the presence of acetate and even SDS. On the other hand, it could also be assumed that both factors were adding to each other. To confirm the influence of SDS on the partitioning behaviour, mixtures of purified pUT649 and RNA were extracted with 40 mM TOMAC in isooctane in the presence of 100 mM NaCl and different SDS concentrations. The organic phase was reextracted with an aqueous phase containing 500 mM NaCl and 10 % (v/v) isopropanol in 10 mM Tris (pH 8.0).

The aqueous phases were analyzed by agarose gel electrophoresis as shown in Figure 55. The general tendency was observed that with an increased concentration of SDS in the feed the amount of contaminating RNA in the aqueous extract was also rising. Therefore, it was decided to precondition the bacterial cleared lysate by diafiltration using a 100 kDa ultrafiltration membrane. This step would allow us to remove the interfering SDS and change the buffer conditions to optimal values. In addition, we expected to remove proteins for the most part that might also have an effect on the partitioning of the nucleic acids. If other contaminants would be depleted as well, could not be predicted and was thus analyzed at a later stage of the project.

In Figure 56 the aqueous raffinates of an extraction of a diafiltered cleared lysate with an organic phase containing 40 mM TOMAC and 0.5 % ethylhexanol in isooctane are shown. The lysate was diafiltered against 10 mM Tris buffer (pH 8.0) and diluted with lithium sulfate solutions in 10 mM Tris (pH 8.0) to achieve defined salt concentrations in the feed.

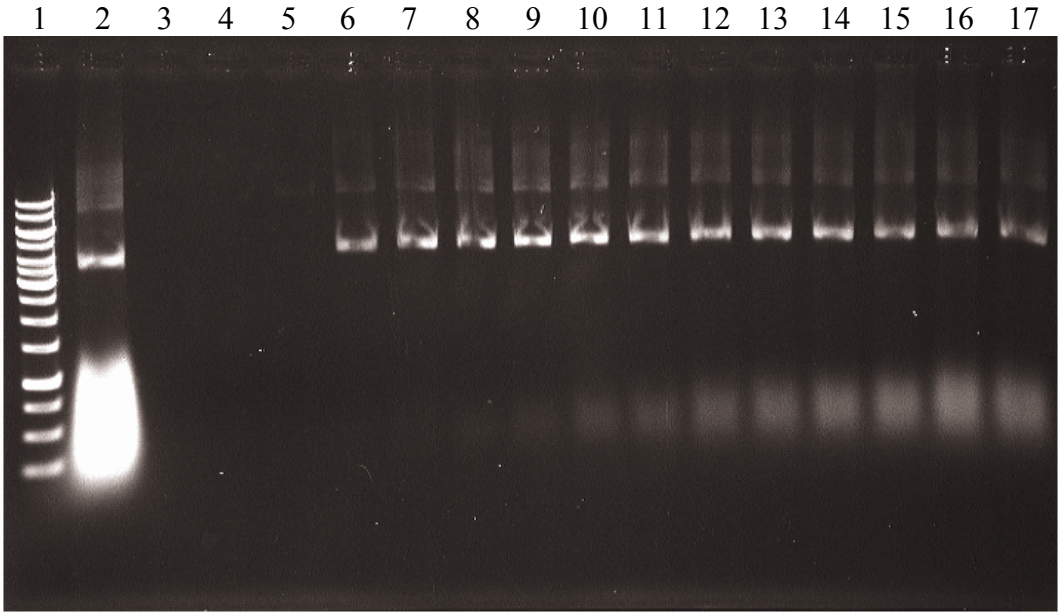


Figure 56 Agarose gel electrophoresis of the aqueous raffinate phase after extraction of a diafiltered bacterial cleared lysate with a reverse micellar phase in the presence of lithium sulfate at different concentrations. Lane 1: DNA 1 kB marker, lane 2: cleared lysate before diafiltration, lanes 3-17: 460-760 mM lithium sulfate in 20 mM steps (Streitner *et al.*, 2007).

At concentrations up to 480 mM Li_2SO_4 all nucleic acids were removed from the aqueous phase (lanes 3 and 4) while at concentrations between 520 and 580 mM Li_2SO_4 only RNA was extracted (lanes 6 to 9). At higher salt concentrations all nucleic acids remained in the aqueous raffinate. This initial attempts showed that this option was certainly able to fulfil our main goal and remove the RNA from the product stream. However, the second option seemed more attractive because a concentration of the product should be possible and the removal of additional contaminants was expected. Therefore, the first purification option was not tested in more details.

Separation of Plasmid Forms by Extraction

Another crucial parameter for plasmid purification is the form distribution in the sample. An ideal purification scheme should be able to remove unwanted oc form or at least deplete it. A minimum requirement for such a process would be that the amount of oc form is not increased during purification. As already indicated in Figure 53 the de-

scribed extraction systems seemed to be capable to selectively extract the desired ccc plasmid form. This observation was further analyzed. To ensure that the two dominant forms observed in agarose gels were ccc monomer and oc form, the starting material was analyzed by capillary gel electrophoresis. With this characterization we were able to use agarose gel electrophoresis as a fast and convenient tool to analyze the distribution of plasmid isoforms in the extraction systems. Figure 57 shows the resulting electropherogram. Most of the plasmid DNA was in the ccc monomer form (83.4 %), while oc forms constituted 14.7 % of the whole plasmid DNA. Ccc dimer forms only made 1.9 % of the preparation and could be disregarded in the following considerations.

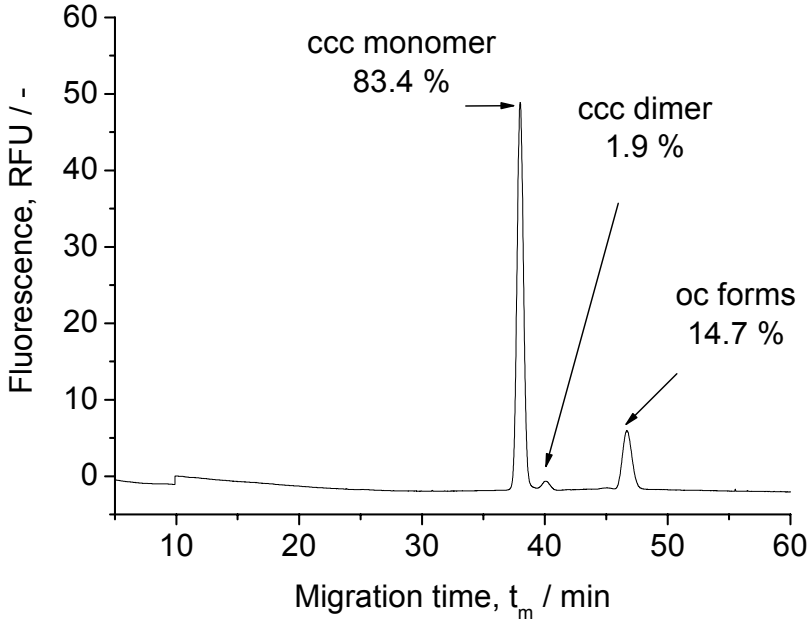


Figure 57 Capillary gel electrophoresis analysis of plasmid pUT649 used as starting material for extraction experiments.

Plasmid DNA was extracted from an aqueous feed phase with LiCl, NaCl, KCl, and KOAc at different concentrations as already described before. The resulting aqueous raffinate phase was analyzed by agarose gel electrophoresis. In Figure 58 the partitioning of the plasmid forms of purified plasmid pUT649 (model system) in water-oil-surfactant systems under the influence of different salts at varying concentrations is shown. In case of LiCl (Figure 58 (A)), the supercoiled form was selectively extracted

at chloride concentrations between 220 and 230 mM. For NaCl the ccc form was extracted at chloride concentrations between 260 and 270 mM and for KCl at chloride concentrations around 290 mM. In the event of potassium acetate a depletion of the ccc plasmid form was also observable at acetate concentrations between 1170 and 1185 mM.

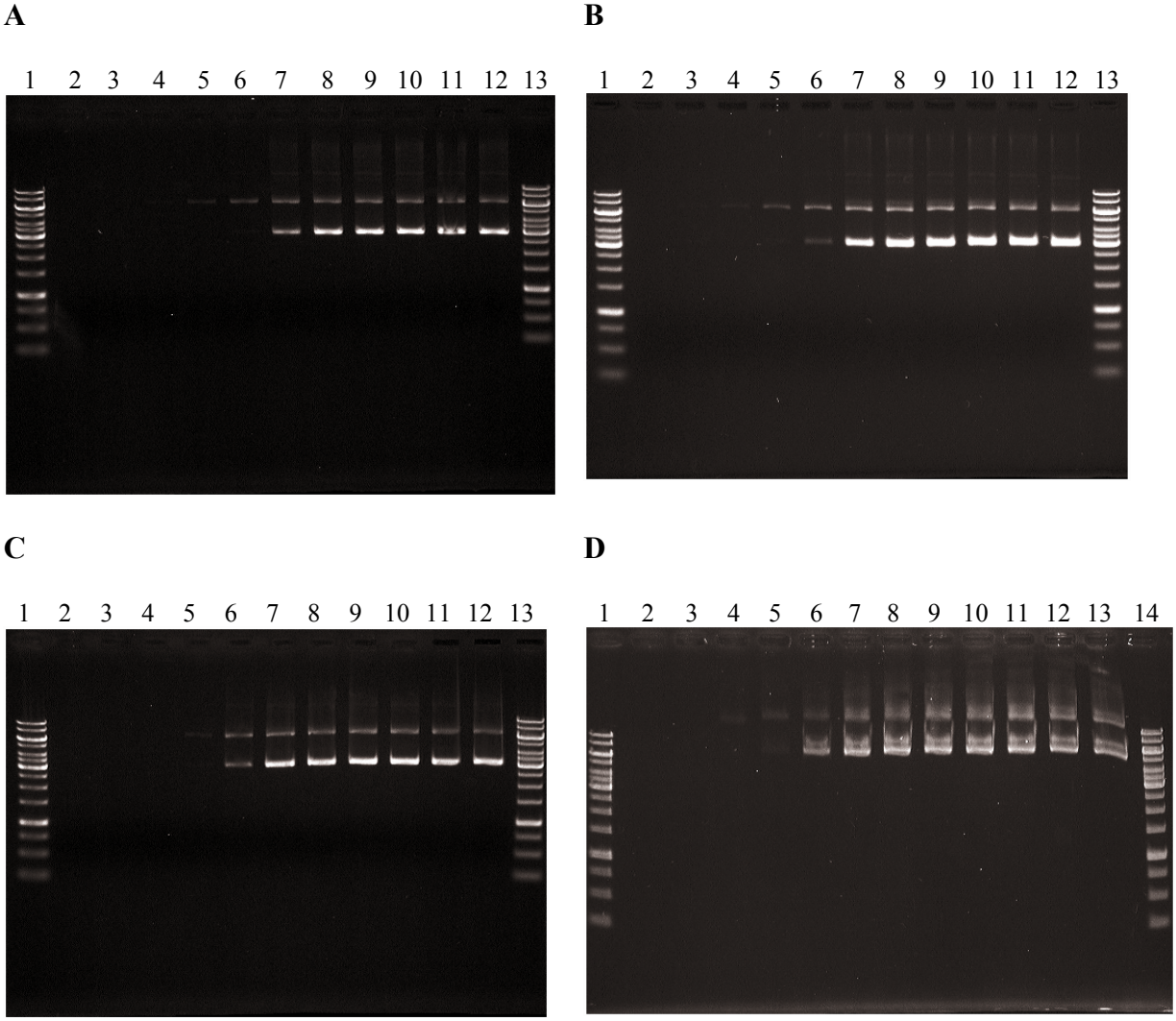


Figure 58 Agarose gel electrophoresis of plasmid pUT649 in the aqueous raffinate after extraction with 40 mM TOMAC and 0.5 % ethylhexanol in isooctane in the presence of alkali metal chlorides (A-C) and potassium acetate (D). Part A: lanes 1, 13: DNA 1 kb marker, lanes 2-12: 190-290 mM LiCl in 10 mM steps. Part B: lanes 1, 13: DNA 1 kb marker, lanes 2-12: 230-330 mM NaCl in 10 mM steps. Part C: lanes 1, 13: DNA 1 kb marker, lanes 2-12: 250-350 mM KCl in 10 mM steps. Part D: lanes 1, 14: DNA 1 kb marker, lanes 2-13: 1140-1305 mM KOAc in 15 mM steps.

In case of lithium sulfate, a selective extraction of the plasmid DNA could also be observed. Although a selective extraction of the supercoiled plasmid DNA form was possible in the model system, it was difficult to accomplished with a diafiltered cleared lysate as feed because surface active compounds, especially proteins, could still be present in sufficient quantity to influence the partitioning behaviour. However, at a small scale we were able to show that it could be accomplished in principle as shown in Figure 59.

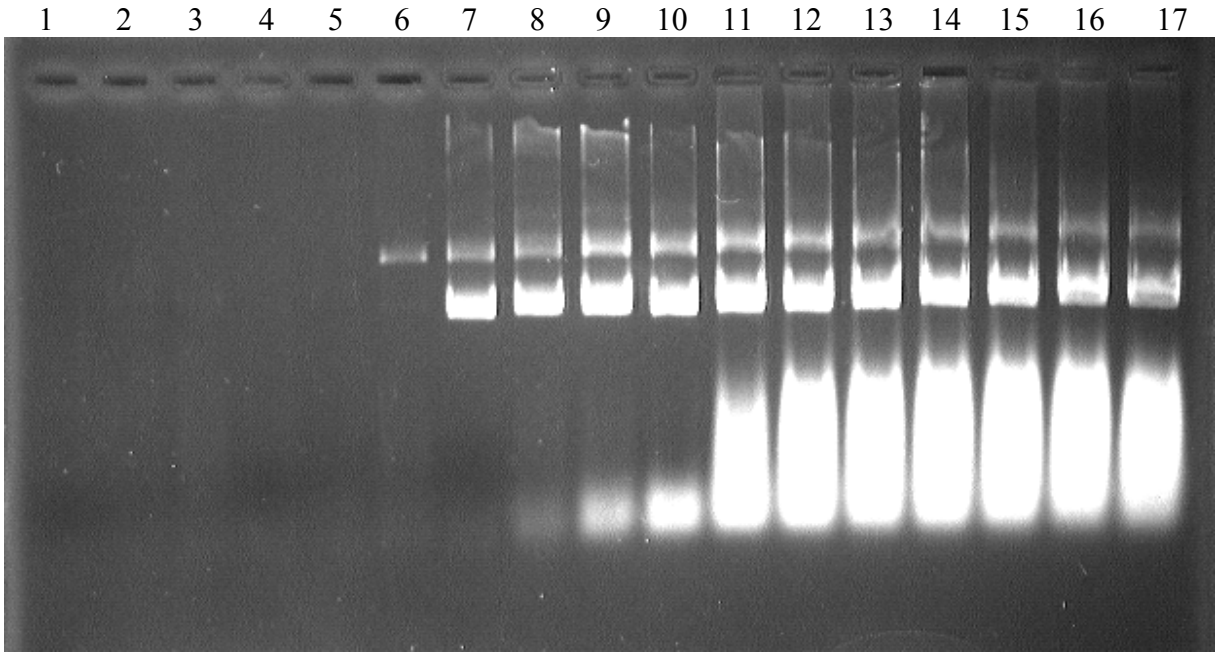


Figure 59 Aqueous raffinate phase after extraction with 40 mM TOMAC and 1.0 % ethylhexanol in isooctane in the presence of different lithium salt concentrations. Lane 1-17: 120-760 mM Li_2SO_4 (40 mM steps).

Nevertheless, in subsequent experiments it was not attempted to find the optimal system with respect to selective extraction of the supercoiled form. In this way a broader salt concentration range could be applied in the forward extraction and even cheap and common salts like NaCl could be applied.

Extractions with Different Plasmids

Extractions with pUC18 (2.7 kB), pUT649 (4.6 kB), and pUK21CMVβ (7.6 kB) from diafiltered bacterial lysates were carried out using a reverse micellar phase comprising 40 mM TOMAC and 0.5 ethylhexanol in isooctane in the presence of lithium sulfate. As shown in Figure 60 the inversion of extraction occured around 480 mM Li_2SO_4 which was in good agreement with the results presented in Figure 54 (A).

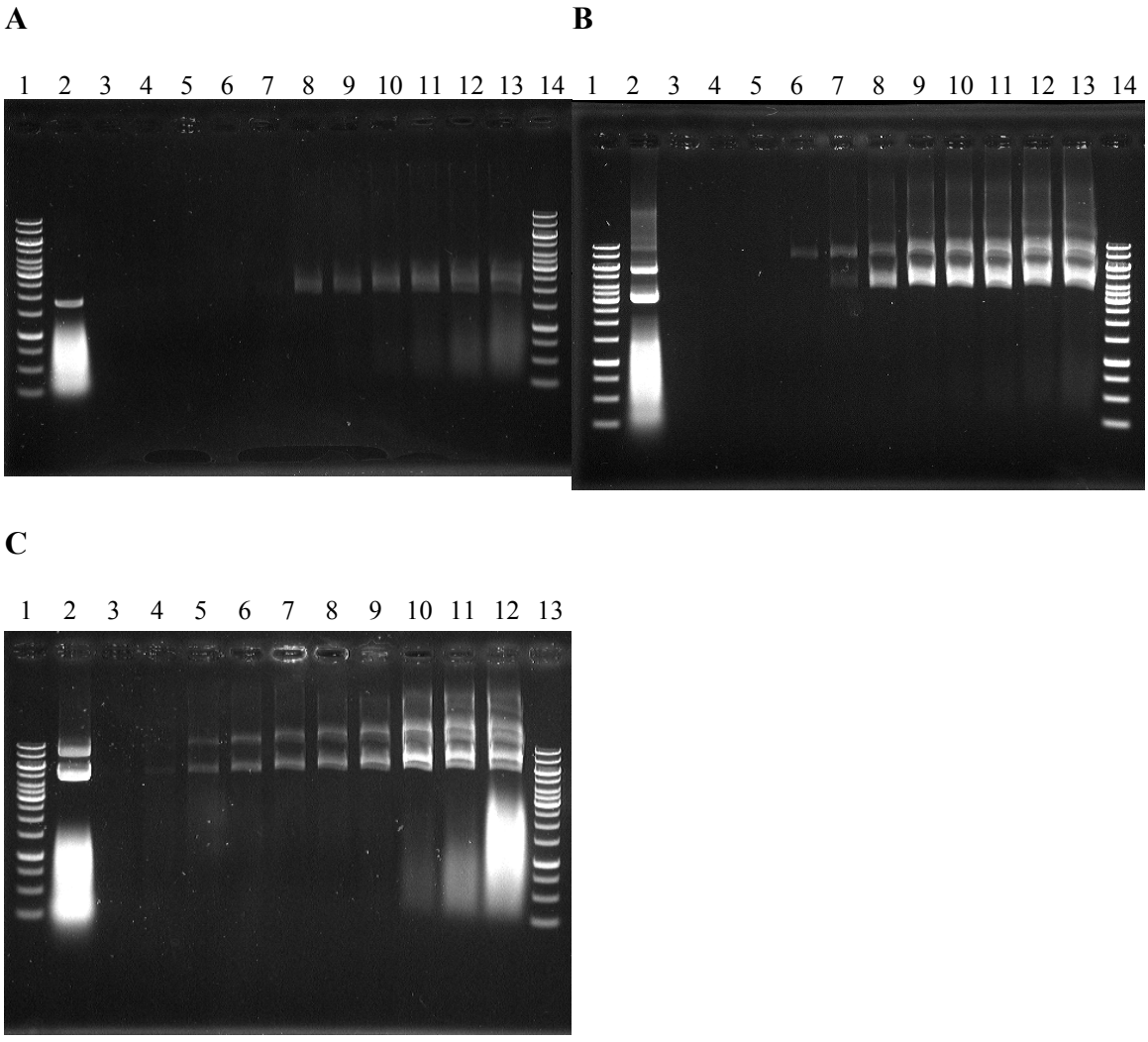


Figure 60 Analysis of aqueous raffinate phases after extraction from a diafiltered bacterial cleared lysate with a reverse micellar phase of 40 mM TOMAC and 0.5 % ethylhexanol in isooctane. A: pUC18. Lane 1, 14: 1 kb marker. Lane 2: diafiltered lysate. Lanes 3 – 13: 360 – 760 mM Li_2SO_4 (40 mM steps). B: pUT649. Lane 1, 14: 1 kb marker. Lane 2: diafiltered lysate. Lanes 3 – 13: 360 -760 mM Li_2SO_4 (40 mM steps). C: pUK21CMVβ. Lane 1, 13: 1 kb marker. Lane 2: diafiltered lysate. Lanes 3 – 12: 40 – 760 mM Li_2SO_4 (80 mM steps).

With the smaller pUC18 the inversion point was located between 520 and 560 mM Li_2SO_4 while the larger plasmid pUK21CMVβ showed an inversion between 80 and 120 mM Li_2SO_4. These results indicated a separation by size.

4.5.2 Forward and Back Extraction

The second process option utilizing forward and backward extraction was considered to be the most convenient one. As already indicated in our initial studies with model systems for the forward extraction, plasmid DNA and RNA could both be removed from the aqueous feed phase.

Effect of Ionic Strength

When applying the reverse micellar extraction mechanism, it could be expected that an increase in the salt concentration should liberate the nucleic acids from the reverse micellar structure.

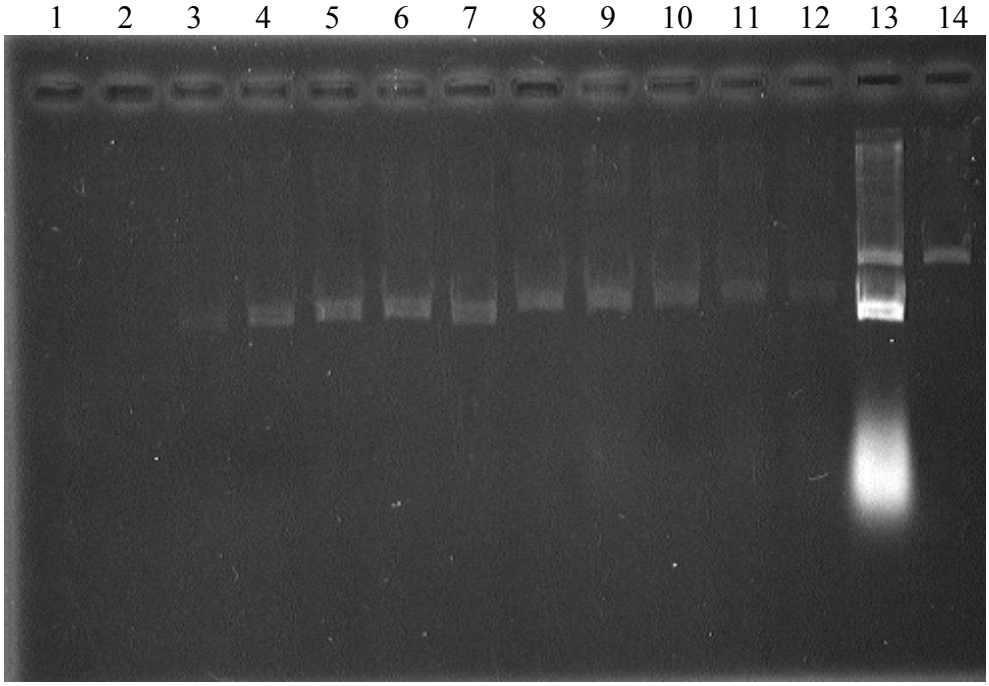


Figure 61 Analysis of aqueous extracts after back extraction of an organic phase with 10 mM Tris buffer (pH 8.0) in the presence of different lithium sulfate concentrations. Forward extraction: Aqueous feed contained 75 mg L^{-1} pUT649, 500 mg L^{-1} RNA and 480 mM Li_2SO_4 which was extracted with 40 mM TOMAC and 0.5 % ethylhexanol in isooctane. Lanes 1-12: Aqueous extract at 520-960 mM Li_2SO_4 (40 mM steps). Lane 13: Aqueous feed. Lane 14: Aqueous raffinate.

However, as can be seen in Figure 61, the recovery of plasmid DNA was very low, although separation from RNA seemed to be very effective. In order to enhance the recovery of plasmid DNA in the back extraction step, other parameters were analyzed in more details which could also influence the partitioning behaviour.

Effect of Alcohol Chain Length and Alcohol Concentration

It was already noted during the examination of the forward extraction process that the alcohol had an effect on the partitioning behaviour. Therefore, it was tested how alcohol concentration and aliphatic chain length would influence the extraction process. During forward extraction it was noted that the alcohol changed the inversion point of the extraction systems. This was also found in case of different alcohols that were added to the back extraction system. Figure 62 shows the analysis of the aqueous extracts that were obtained after back extraction in the presence of 2-propanol (A) and 1-hexanol (B).

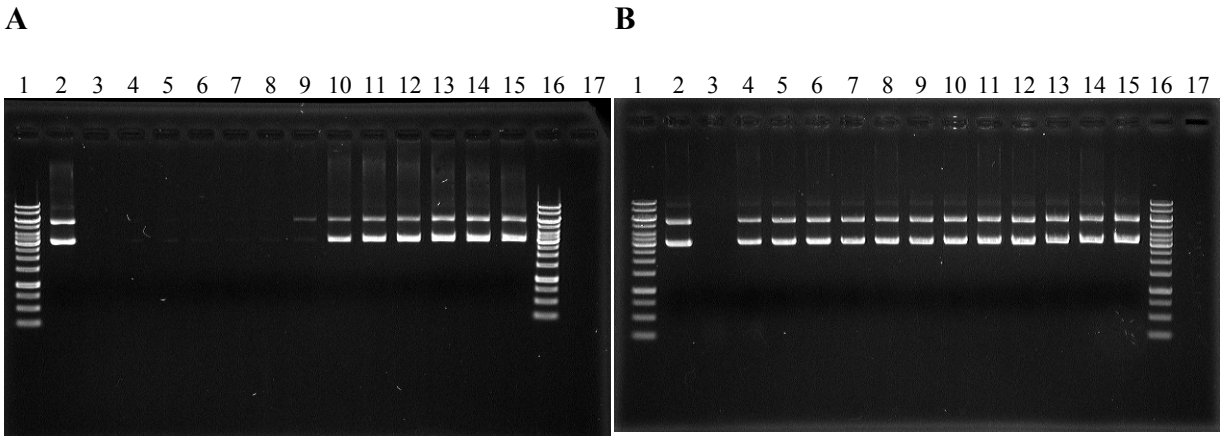


Figure 62 Change of the inversion of partitioning when using different alcohols. A: Extraction in the presence of 2-propanol. B: Extraction in the presence of 1-hexanol. Extraction conditions: Aqueous feed contained 100 mM NaCl, 50 mg L^{-1} pUT649 in 10 mM Tris buffer (pH 8.0) and was extracted with 40 mM TOMAC and 2-propanol (A) or 1-hexanol (B) in isooctane. The organic phase was reextracted with an aqueous phase that contained different NaCl concentrations and that was supplemented with 5 % (v/v) 2-propanol (A) or 1-hexanol (B). Lanes 1,16: 1 kB DNA marker. Lane 2: Aqueous feed. Lane 3: Aqueous raffinate. Lanes 4-15: Aqueous extract in the presence of 40-480 mM NaCl (40 mM steps).

In case of 2-propanol, the plasmid DNA was reextracted from the organic phase at NaCl concentrations above 320 mM and at concentration above 80 mM NaCl when 1-hexanol was present in the extraction system. In addition, an enhanced recovery was observed for this back extraction procedure when compared to the results presented in Figure 61 where no alcohol was added during back extraction. It could be concluded that plasmid DNA could easily be reextracted from the organic phase by increasing the alcohol fraction in the back extraction step and that the salt concentration did not play a predominant role here.

As a next step it had to be analyzed how the alcohols effect the separation of plasmid DNA from RNA. For this purpose a diafiltered cleared lysate was extracted with an organic phase that contained 40 mM TOMAC and 0.5 % ethylhexanol (Figure 63 (A)) or butanol (Figure 63 (B)) in isooctane in the presence of 200 mM lithium sulfate. Back extraction of the nucleic acids from the organic phase into a new aqueous reception phase was accomplished by the addition of 4 % butanol in the presence of lithium sulfate at different concentrations.

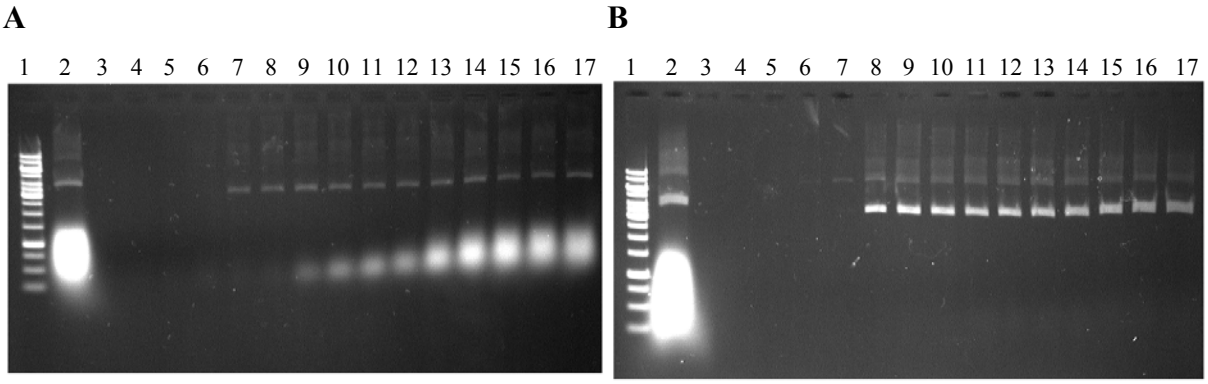


Figure 63 Separation of plasmid DNA from RNA in the presence of different alcohols during forward extraction. A: 0.5 % (v/v) ethylhexanol. B: 0.5 % (v/v) butanol. Aqueous extract phases achieved after back extraction were analyzed by agarose gel electrophoresis. Conditions for backward extraction: 4 % (v/v) butanol and 120 – 400 mM Li_2SO_4 in 40 mM steps.

With a longer chain alcohol like ethylhexanol in the forward extraction system, significant amounts of RNA were reextracted along with the plasmid DNA during back extraction in the presence of the shorter alcohol butanol. When in comparison butanol was also used during forward extraction, no significant amount of RNA was reextracted. In

both cases the recovery of plasmid DNA seemed to be unaffected by the alcohol in the forward extraction system.

From these results it was concluded that the extraction was predominantly influenced by the alcohol concentration. How the alcohol affects the extraction systems was still unknown and it could only be speculated here. More detailed results were expected from structural analysis of the extraction systems which are currently performed with an external partner. These results will hopefully shed more light on the mechanism of extraction and allow us to evaluate the effects described here from that perspective.

As the next step, the recovery of plasmid DNA during the extraction procedure had to be examined more thoroughly. Although the recovery seemed good when looking at the agarose gels, a more quantitative analysis was advisable. Since it was found out that the alcohol was the main influencing parameter for nucleic acid extraction, the recovery of plasmid DNA was analyzed in the presence of different alcohols. For this purpose, the plasmid concentration in a bacterial cleared lysate was determined by UV absorption after purification with QIAGEN tips. The DNA concentrations in the aqueous extracts were determined in the same way. The results are summarized in Figure 64.

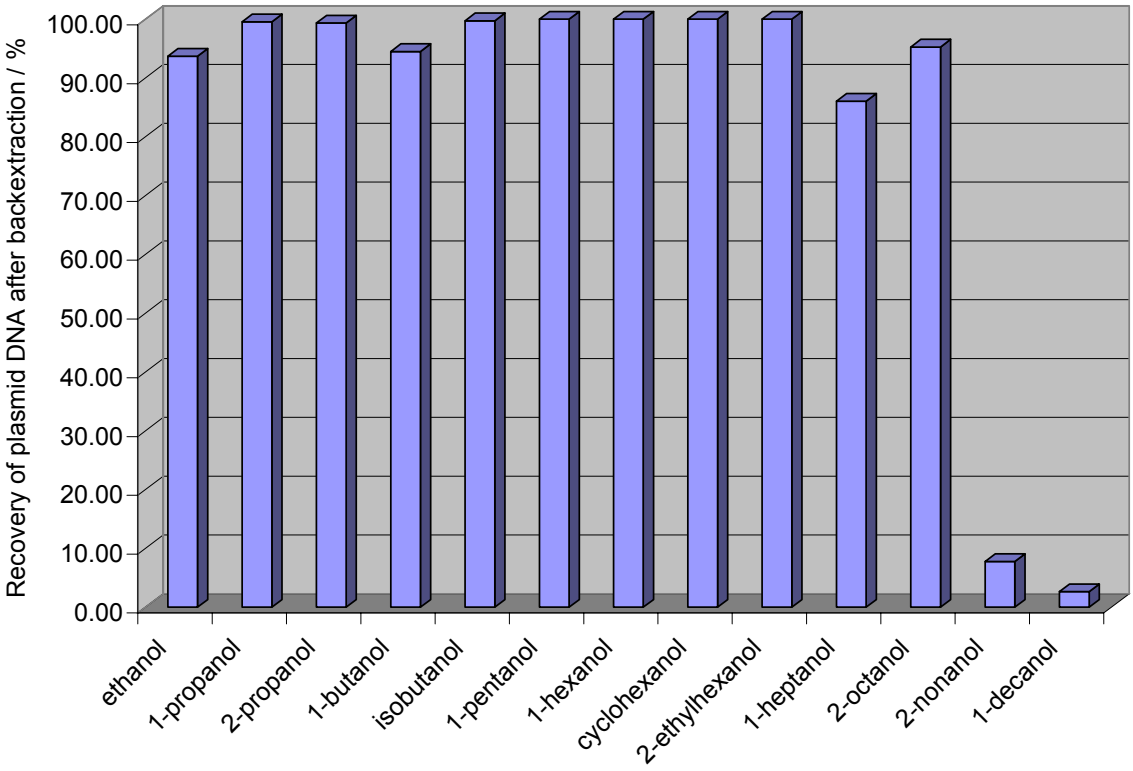


Figure 64 Recovery of plasmid DNA after back extraction in the presence of different alcohols. Forward extraction: 100 mM NaCl, 0.04 – 0.2 % (v/v) alcohol. Back extraction: 500 mM NaCl, 10 % (v/v) alcohol.

As can be seen, in most cases the recoveries were above 90 %. Only in the case of 1-nonanol and 1-decanol a significantly low recovery below 10 % was observed.

From these results, an extraction process was designed. First a bacterial cleared lysate was diafiltered against 50 mM NaCl in 10 mM Tris (pH 8.0) that was used as aqueous feed. Both RNA and plasmid DNA were extracted from this feed by an organic phase comprising 40 mM TOMAC and 0.05 % 2-propanol in isooctane. Plasmid DNA was reextracted from this phase into a new aqueous phase containing 500 mM NaCl in 10 mM Tris (pH 8.0). This procedure was further analyzed with respect to the depletion of the major contaminants in the final product. The results from this process are summarized in Table 23.

Table 23 Depletion of contaminations during the extraction process. Values in the extract fulfilling the specifications are marked green, those exceeding them are marked red.

	$x_{(protein/pDNA)}$ / mg g^{-1}	$x_{(LPS/pDNA)}$ / E.U. mg^{-1}	$x_{(chr.DNA/pDNA)}$[a] / mg g^{-1}
Lysate	15 000	145 500[b]	590
Feed	478	291 000	469
Raffinate	not detectable	n/a	n/a
Extract	not detectable	3 400	1.8

[a] Values based on continuous lysis and froth flotation.
[b] Calculated on the basis of the measurement in the aqueous feed.

Protein concentrations were already reduced during diafiltration and subsequently removed by the extraction. In this way, no protein was detectable in the aqeuous raffinate or extract. With respect to chromosomal DNA, no significant depletion was observed during diafiltration which was assumed due to the cut off of the membrane applied in this process. However, the extraction process was capable to reduce the amount of chromosomal DNA sufficiently. Endotoxins were not completely depleted by reverse micellar extraction and the concentration in the aqueous raffinate exceeded the specifications.

The concentration of chromosomal DNA in bacterial lysates prepared by different methods has already been described in a preceding chapter. It was considered to be most interesting if reverse micellar extraction would be capable of removing this contamination. The results in Figure 65 show that the chromosomal DNA got sufficiently depleted. Although the value obtained after mixing with 200 rpm was comparably high, it was still within the specifications set for pharmaceutical plasmid DNA.

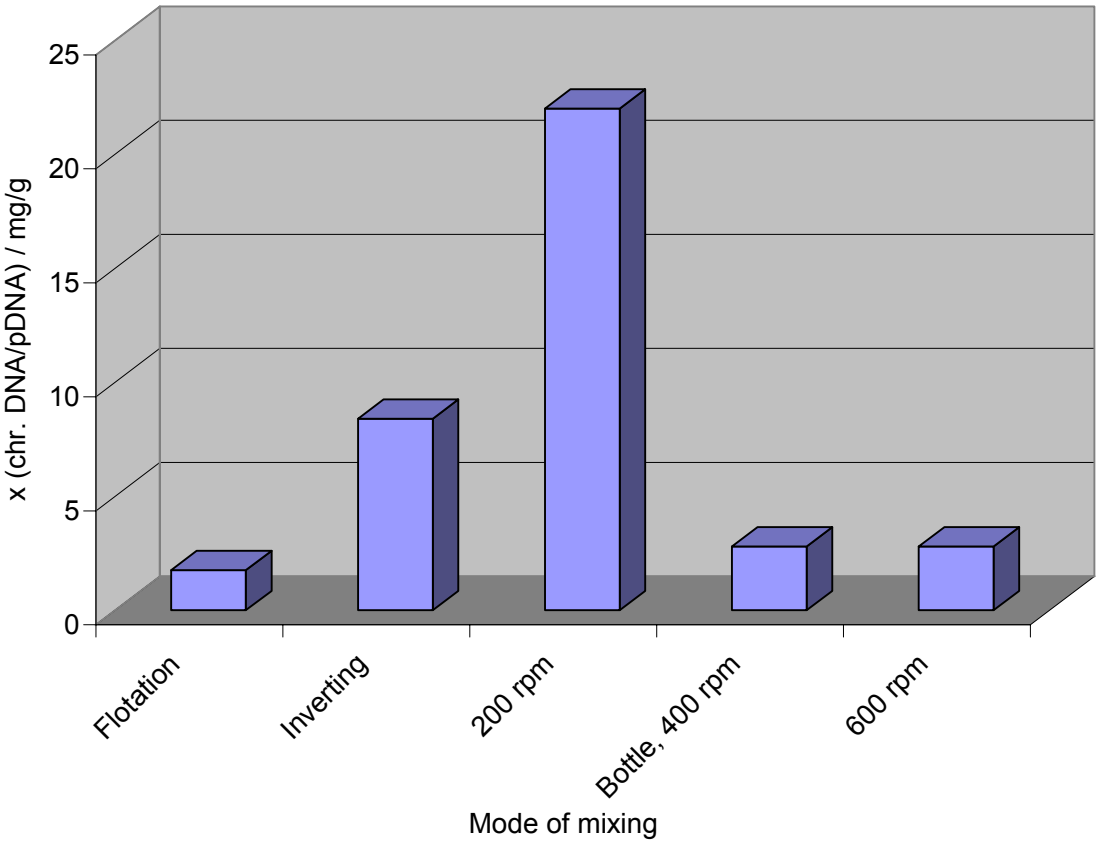


Figure 65 Concentration of genomic DNA relative to plasmid DNA in the extract after extraction with reverse micellar two-phase system (Tschapalda, 2008).

The concentration of the surfactant has been determined in the aqueous extract obtained after back extraction by titration experiments. With 1 mmol L^{-1} this concentration was considerably low, but probably not tolerable for a pharmaceutical product. The experiments dealing with process integration that is described in more details in the subsequent section showed that TOMAC could be efficiently removed by a diafiltration step following the extraction process. On the contrary, anion exchange chromatography with Fractogel DEAE that was applied for subsequent purification and concentration of the plasmid DNA was not capable of depleting TOMAC to levels below the detection limit. It could be assumed that TOMAC bound to the chromatographic carrier matrix by hydrophobic interaction. In conclusion, other chromatographic materials based on different carriers are probably better suited for this purpose.

Additionally, the capacities of the reverse micellar phases for pure nucleic acids have been determined and were found to exceed values of 2 mg mL^{-1}. However, in case of a complex sample like a cleared lysate it was found that this capacity could not be utilized comletely due to the concentrations of the contaminants.

4.5.3 Integration of Extraction into Different Purification Processes

The capability of the extraction process for the separation of plasmid DNA from RNA has been demonstrated in the previous chapters. In addition, other crucial contaminants could be depleted very effectively as shown in Table 23. Only the endotoxin concentration exceeded the desired purity level. Therefore, the extraction procedure was integrated into several different purification schemes taking the availability of special equipment into account. These process options are summarized in Figure 66 and will be discussed in the following paragraphs.

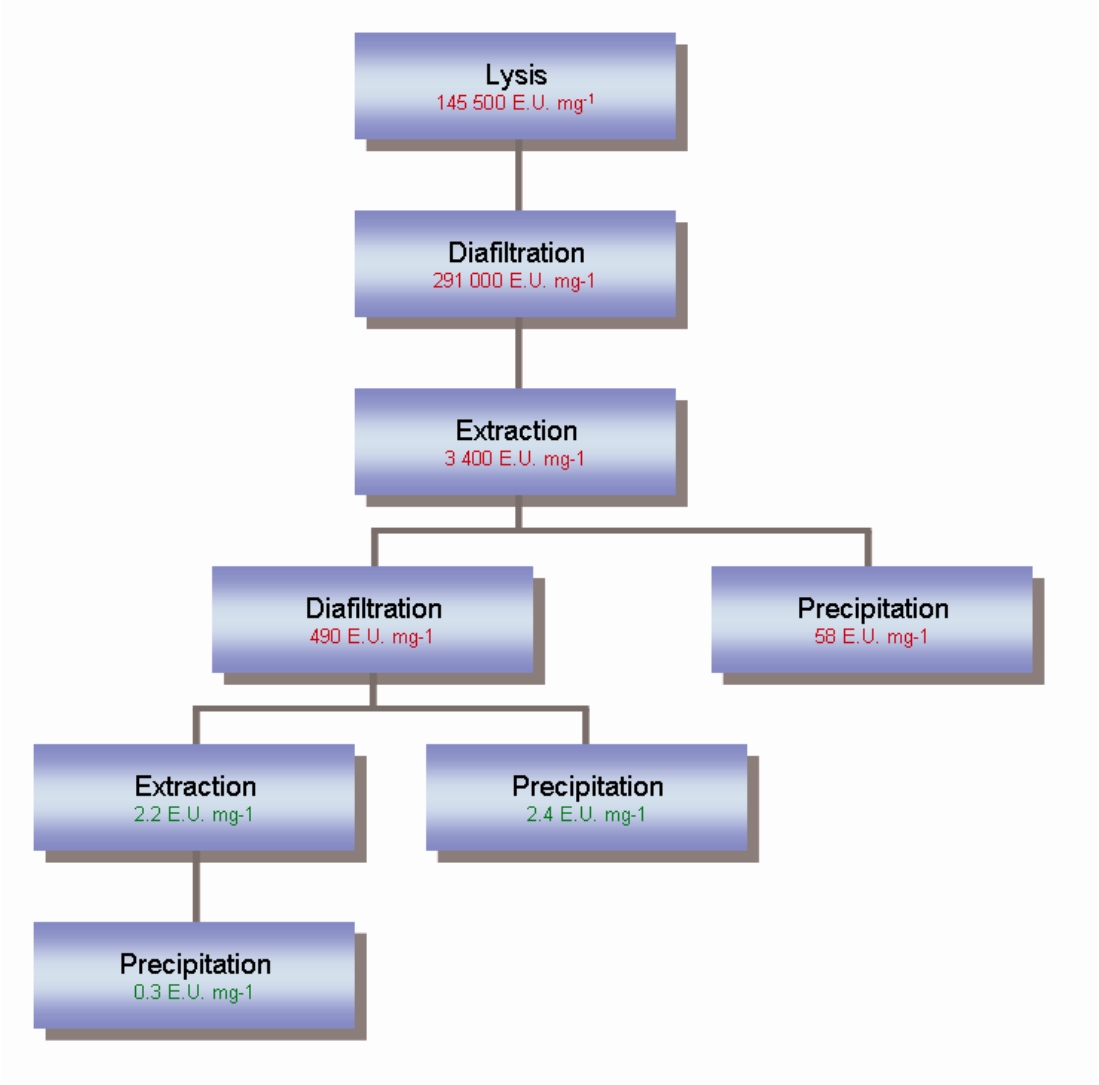


Figure 66 Process options for pharmaceutical grade plasmid production utilizing the developed extraction system and the endotoxin levels determined for each process step (values meeting the specification are green).

First, a diafiltered lysate has been subjected to the previously described extraction procedure. The resulting extract was purified further as shown in Figure 66 and analyzed with respect to LPS depletion.

Endotoxin Removal

As already outlined before, the LPS concentration in the extract reached values of 3400 E.U. mg^{-1}. Precipitation of the plasmid DNA with 2-propanol was considered to be a reasonable option for further concentration and buffer exchange at the milligram to

gram production scale because a significant concentration due to volume reduction could be achieved during back extraction. Using this process option, endotoxin levels were reduced to 58 E.U. mg^{-1} in the final product which was above the specification of 10 E.U. mg^{-1}. For large scale production, precipitation could be substituted by diafiltration. A sufficient number of diafiltration cycles should accomplish complete LPS removal. Unfortunately, endotoxin concentrations still exceeded the desired value with 490 E.U. mg^{-1} after 4 diafiltration cycles. The reason for the high endotoxin concentration after diafiltration was that the LPS molecules have surfactant like properties and form larger structures like micelles or vesicels that were held back by the ultrafiltration membrane and were in this way partially concentrated. One solution for this problem is the addition of a surfactant that will destroy the LPS structures. Triton X-114 was found to be suitable for this purpose. However, this surfactant has to be removed from the product stream which could only be accomplished by extensive diafiltration with endotoxin free buffers. These simple process schemes were however well suited for the production of research grade plasmid DNA that can be used in preclinical applications. Another option is to exchange the buffer after extraction by diafiltration and concentrate the sample. Subsequent precipitation with 2-propanol resulted in high quality plasmid DNA with low levels of LPS (2.4 E.U. mg^{-1}) in the final product. Alternatively, the second diafiltration could be succeeded by a second extraction process. It was shown that this step sufficiently depleted the endotoxins to a concentration of 2.2 E.U. mg^{-1} below the desired level. Additional precipitation with 2-propanol resulted in an even lower concentration of LPS with 0.3 E.U. mg^{-1}. When comparing this system with other extraction processes as shown in Table 24, the reverse micellar extraction process was found to be superior with respect to product recovery and the depletion of proteins and chromosomal DNA. With respect to LPS removal results have only been presented by Trindade *et al.* (2005) being comparable to our values. It should be noted, that in case of the other extraction systems not every quality parameter has been determined.

Table 24 Comparison of extraction processes.

Extraction system	Plasmid recovery / %	RNA removal / %	x(protein/pDNA) / mg g^{-1}	x(chr.DNA/pDNA) / mg g^{-1}	x(LPS/pDNA) / E.U. mg^{-1}	Reference
PEG-600/phosphate	42	100	n.d.	5[a]	n/a	Ribeiro *et al.*, 2002
PEG-600/$(NH_4)_2SO_4$	75	100	395	n/a	3 646 (n.d.[b])	Trindade *et al.*, 2005
PEG-800/phosphate	60 – 75	89	n/a	n/a	n/a	Frerix *et al.*, 2005
$EO_{50}PO_{50}$/dextran	n/a	80	6 522	n/a	n/a	Kepka *et al.*, 2004
TOMAC/isooctane/H_2O	100	100	n.d.	1.8	3 400 (2.4[c])	this thesis

[a] Value determined with Southern blot.
[b] After subsequent purification with HIC.
[c] After additional diafiltration (4 cycles) and 2-propanol precipitation.

Depletion of Open Circular Plasmid Forms

Although the extraction process was capable to selectively extract the supercoiled plasmid form when working with a model system, it was found that this was difficult to achieve when working with a diafiltered lysate. For this reason, a further optimization in this direction was not attempted. Instead, another process option is presented. Plasmid DNA was reextracted from the organic phase into 2.5 M ammonium sulfate buffer. This extract was applied to a Plasmid Select (GE Healthcare) column being capable of accumulating the supercoiled form as can be seen in the chromatogram presented in Figure 67.

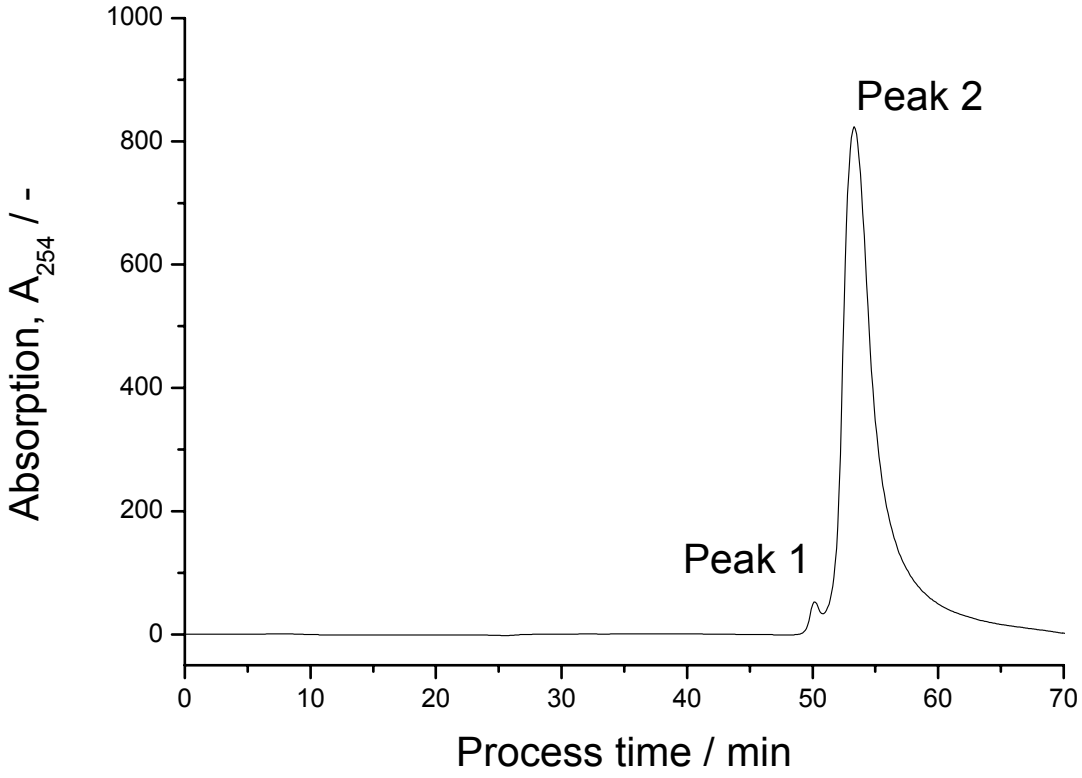


Figure 67 Chromatogram of an extract phase with 2.5 M ammonium sulfate that has been applied to a Plasmid Select (GE) column. Elution was performed using a gradient from 2.5 M ammonium sulfate to water over 11 column volumes.

The depletion of the open circular form was verified by capillary gel electrophoresis as shown in Figure 68.

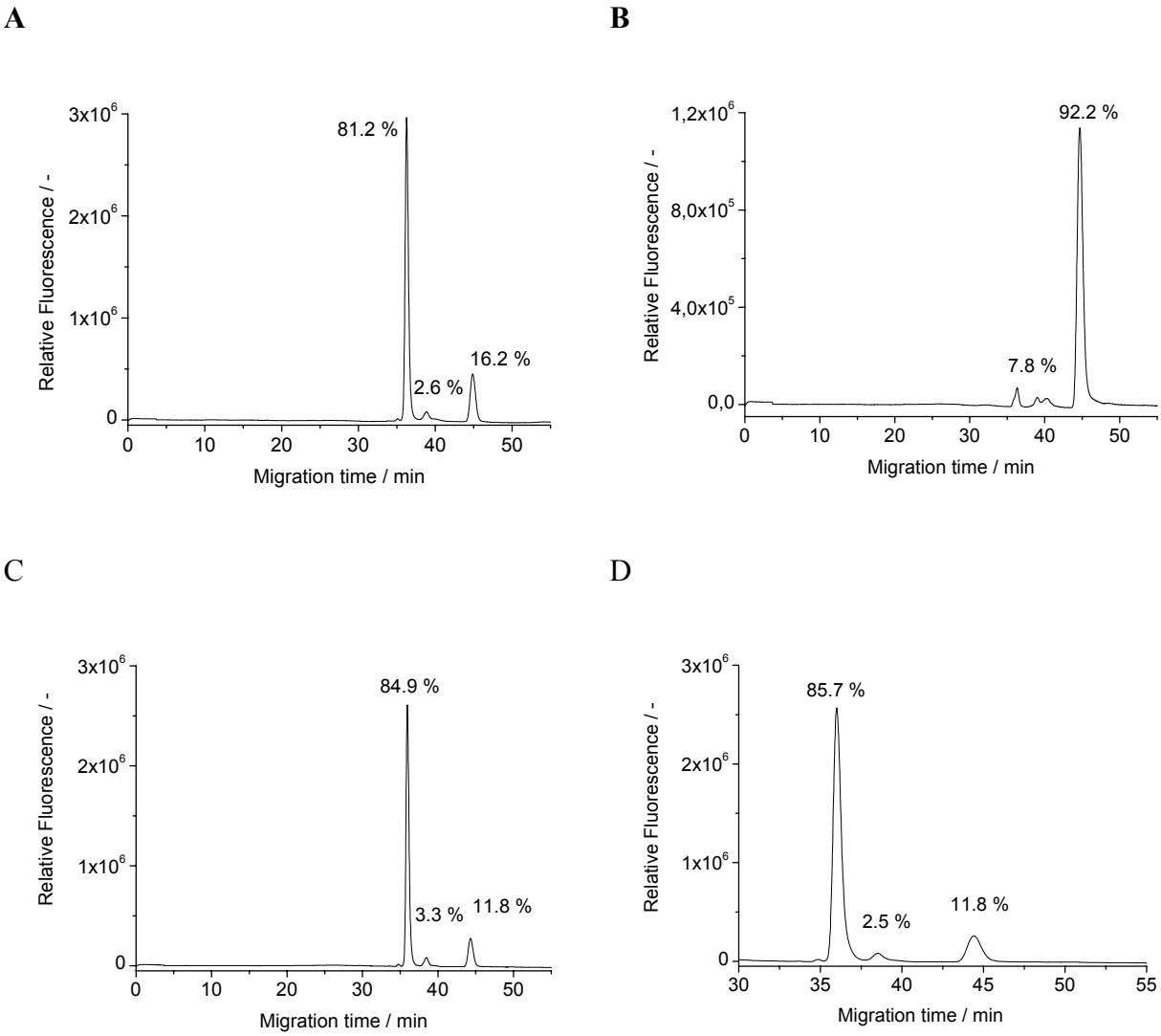


Figure 68 CGE analysis of the feed fraction (A) and the eluted peaks from Plasmid Select chromatography. Part B shows the electropherogram of the first elution peak while part C shows the electropherogram of the second peak. Part D shows the electropherogram of the sample eluted from subsequent anion exchange chromatography on Fractogel DEAE. Analysis was performed as described by Schmidt *et al.* (1999b)

The extract phase could be applied to the column without further modification. In comparison to Lemmens *et al.* (2003) binding was accomplished at higher conductivity because the conditions for selective capture of the supercoiled form were difficult to

achieve. Therefore, oc and ccc form were loaded to the column and eluted separately. In this way, the amount of ccc form could be increased and the oc form was depleted in the first elution peak. However, in this example the product did not meet the quality requirements set for pharmaceutical plasmid DNA with respect to plasmid form distribution. Better results can be expected when using a starting material that shows a larger fraction of ccc form or by optimizing the Plasmid Select step for a complete removal of the oc form. Further downstream processing could be achieved with an additional anion exchange chromatography. It has to be noted that this option was not tested with respect to LPS depletion. However, from results published in the literature (Lemmens *et al.*, 2003) it could be assumed that the endotoxins could be removed during the chromatographic steps. In a manner of speaking, this process option is closely related to the process described by Lemmens *et al.*, only the size exclusion step is substituted by an extraction process. With respect to productivity and scalability, the extraction variant is far superior to gel filtration.

Application of a Diluted Lysate

If no diafiltration is available, this unit operation could be avoided by simply diluting the cleared lysate with the same volume of water. In this way, the high concentrations of acetates present would be reduced as well as the influence of surface active compounds. As can be seen in Figure 69 the diluted bacterial lysate contained a large amount of RNA with a broad size distribution (lane 2). After extraction with a reverse micellar phase no nucleic acids could be detected in the raffinate (lane 3). Subsequent back extraction with a new aqueous phase recovered the plasmid DNA along with a minimum of contaminating RNA (lane 4) which could be removed by anion exchange chromatography. The eluted fractions of such a polishing step using Fractogel DEAE are shown in lanes 5 to 8. As already mentioned before, this process option has also not been analyzed for its ability to remove endotoxins, but it can be assumed that the chromatographic procedure will deplete them sufficiently.

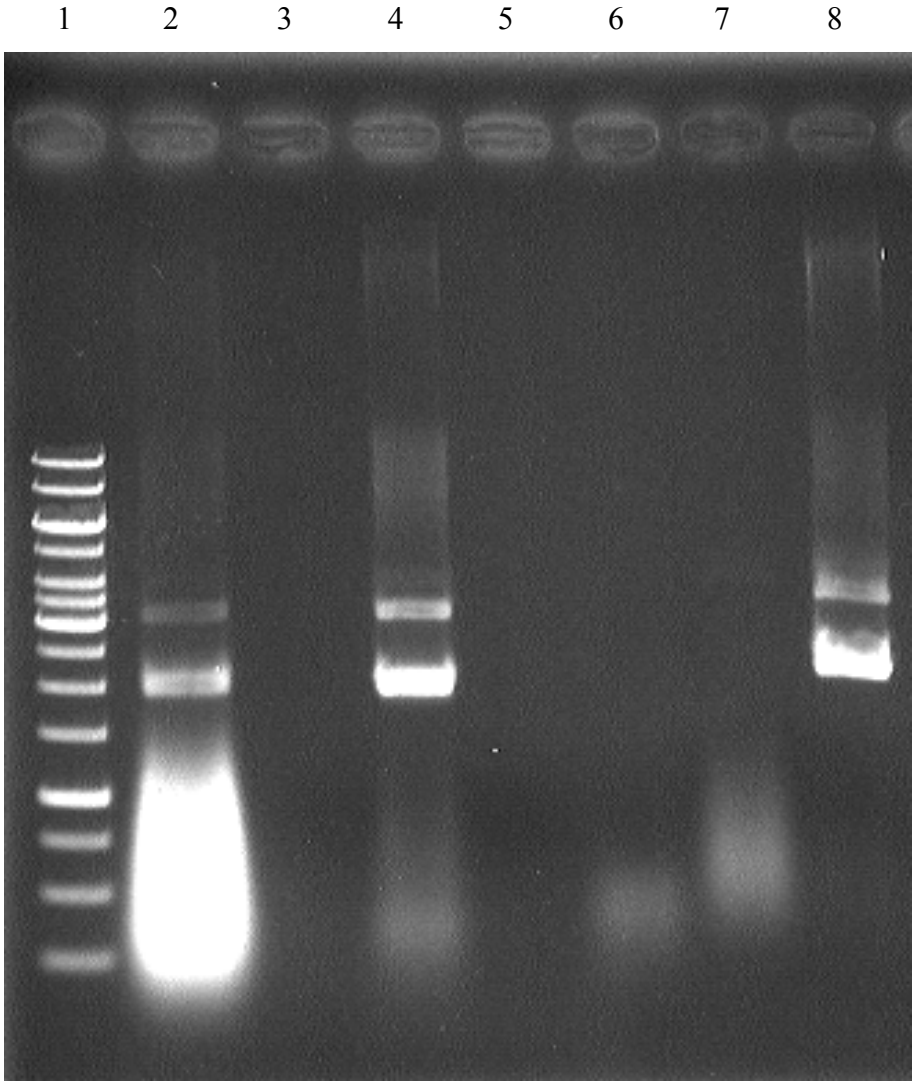


Figure 69 Analysis of an extraction process using a diluted bacterial cleared lysate as feed. Forward extraction: bacterial lysate was diluted with the same volume of water and extracted with 40 mM TOMAC and 0.1 % ethylhexanol in isooctane. Back extraction: Aqueous solution with 500 mM NaCl and 4 % butanol. Lane 1: 1 kb marker. Lane 2: cleared lysate. Lane 3: aqueous raffinate phase after forward extraction. Lane 4: aqueous extract phase after back extraction. Lane 5: flow through of anion exchange chromatography on Fractogel DEAE (Merck). Lane 6, 7: eluted RNA fractions. Lane 8: eluted pDNA fraction.

Nevertheless, the chromatographic step could be optimized further with respect to endotoxin removal by the addition of Triton X-114 to the chromatographic buffers. This will again destroy LPS aggregates which will then be removed during column wash.

4.5.4 Conclusions

By determining the conditions for selective extraction of RNA and plasmid DNA using model systems, we have been able to remove the RNA from a diafiltered cleared lysate. Additionally, both species could be extracted from the aqueous feed and plasmid DNA could completely be recovered in a new recipient phase by the addition of aliphatic alcohols in appropriate concentrations.

This two step extraction procedure has been characterized with respect to the depletion of proteins, chromosomal DNA, and LPS. Depletion was very good, but the product quality achieved did not completely meet the specifications for pharmaceutical plasmid DNA because LPS levels were above specification. In comparison to other extraction systems, the reverse micellar two-phase separation was equally effective or even superior in all cases. However, it should be noted, that in all cases subsequent purification was necessary to achieve the desired product quality. Other advantages of the extraction system were the short processing time of only a few minutes for complete partitioning and the capability to concentrate the product stream substantially.

The extraction procedure was integrated into different process schemes. Several process options were outlined that resulted in essentially pure plasmid DNA that met the specifications. Additionally, process options that allowed the selective purification of the ccc form as well as the application of a diluted lysate have been presented.

With respect to the mechanism of the extraction process, a separation by size could be assumed. Although commonly known spherical reverse micelles only have a size of a few nanometers (5 -50 nm depending on the surfactant and solvent), structures formed by TOMAC and other surfactants in isooctane were capable to accomodate the comparably large plasmid DNA. Earlier experiments by other researchers showed that reverse micellar phases of AOT incorporating nucleic acids consisted of three major populations with the size of 5 nm, 100 nm, and 1000 nm (Pietrini and Luisi, 2002). The larger structures were very unstable while the 100 nm structures accomodated condensed and normal DNA. It is assumed that the larger structures are clusters of smaller reverse micelles. In this way, an incorporation of the plasmid DNA by cationic reverse micelles in our experiments could probably be explained by such structures too. On the other hand, rod like structures that can even form networks of reverse micelles can also accomodate

plasmid DNA. Research with respect to the structure and the extraction mechanism is currently performed.

4.6 Stability of Plasmid DNA during Storage and Application

The stability of biomolecules is a serious issue that has been occassionally addressed in the scientific literature. With respect to plasmid DNA used in gene medicine the stability of the nucleic acid during storage and application is of major interest. The supercoiled form will be nicked usually by irradiation with UV light, enzymatic activity, or chemical hydrolysis resulting in increased amounts of open circular form when only one strand break occurs. In case of a double strand break the linear plasmid form is also observed. Further, the plasmid DNA can be continuously degraded by chemical or enzymatic hydrolyis resulting in decreasing concentrations of DNA.

4.6.1 Plasmid Stability during Application

Direct injection of plasmid DNA has become an established technology for the delivery of naked DNA into target tissue. In case of jet injection or particle bombardment the structural stability of the plasmid DNA during injection is expected to be a crucial parameter for successful expression of the therapeutic genes. Therefore, plasmid DNA was subjected to a jet injection procedure at different injection pressures and the plasmid isoform distribution was analyzed by capillary gel electrophoresis as described in Walther *et al.* (2002). Figure 70 shows that the plasmid DNA got degraded by this procedure and the amount of oc form rose with increased injection pressure. Although at pressures < 2.5 bar the degradation of the supercoiled form was minimal, the gene expression in colon carcinoma in nude mice was rather low. At higher injection pressures an increased expression level was observed. This was due to the fact that at low pressures the tissue was only penetrated for 4-6 mm (2.0 bar) or 5-7 mm (2.4 bar). Therefore, a compromise had to be made between good penetration of the tissue and reduced structural stability of plasmid DNA during injection. The correlation between structural stability and expression levels in target tissue was demonstrated when analyzing the storage stability of plasmid DNA at low temperatures (-80 °C) and during storage in the refrigerator (4 °C) (Walther *et al.*, 2003).

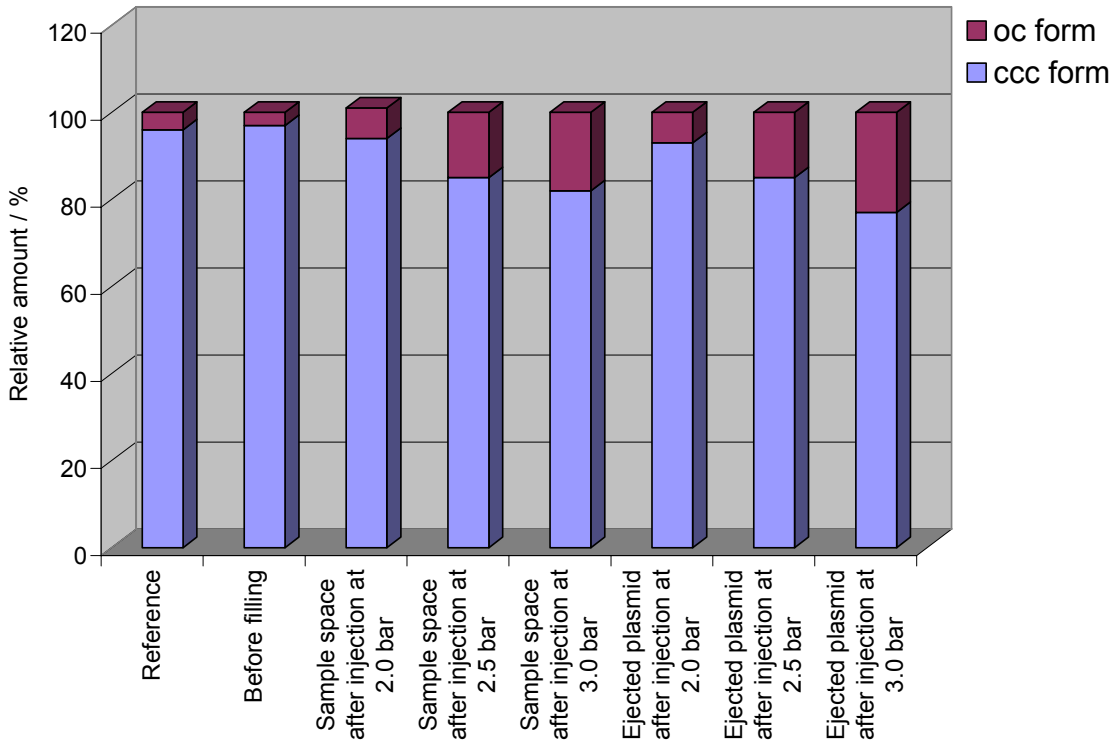


Figure 70 Stability of plasmid DNA during jet gun injection (Walther *et al.*, 2002).

Here, the stability of plasmid DNA under these conditions was analyzed by capillary gel electrophoresis over a period of 13 month. At -80 °C the plasmid DNA remained intact and no change in the form distribution was observed as can be seen in Figure 71 (A). Degradation of the supercoiled form and an increasing amount of open circular form could be observed after 6 month storage at 4 °C. Degradation continued almost linearly and after 9 month of storage even linear plasmid DNA could be observed by CGE analysis. Finally, plasmid DNA stored for 1, 2, and 13 month at -80 and 4 °C was injected into xenotransplanted human colon carcinoma of nude mice and expression of the reporter protein was monitored as shown in Figure 72. Expression of LacZ following transfection with the plasmid DNA stored at -80 °C resulted in rather constant expression levels of 12000 µg g^{-1} (average value of LacZ relative to total protein). On the contrary, the tissue transfected with the plasmid stored at 4 °C resulted in a decreasing LacZ expression. After 13 month of storage an expression level of 330 µg g^{-1} was observed. These results showed that structural stability of the supercoiled plasmid DNA is a prerequisite for efficient gene expression after jet injection.

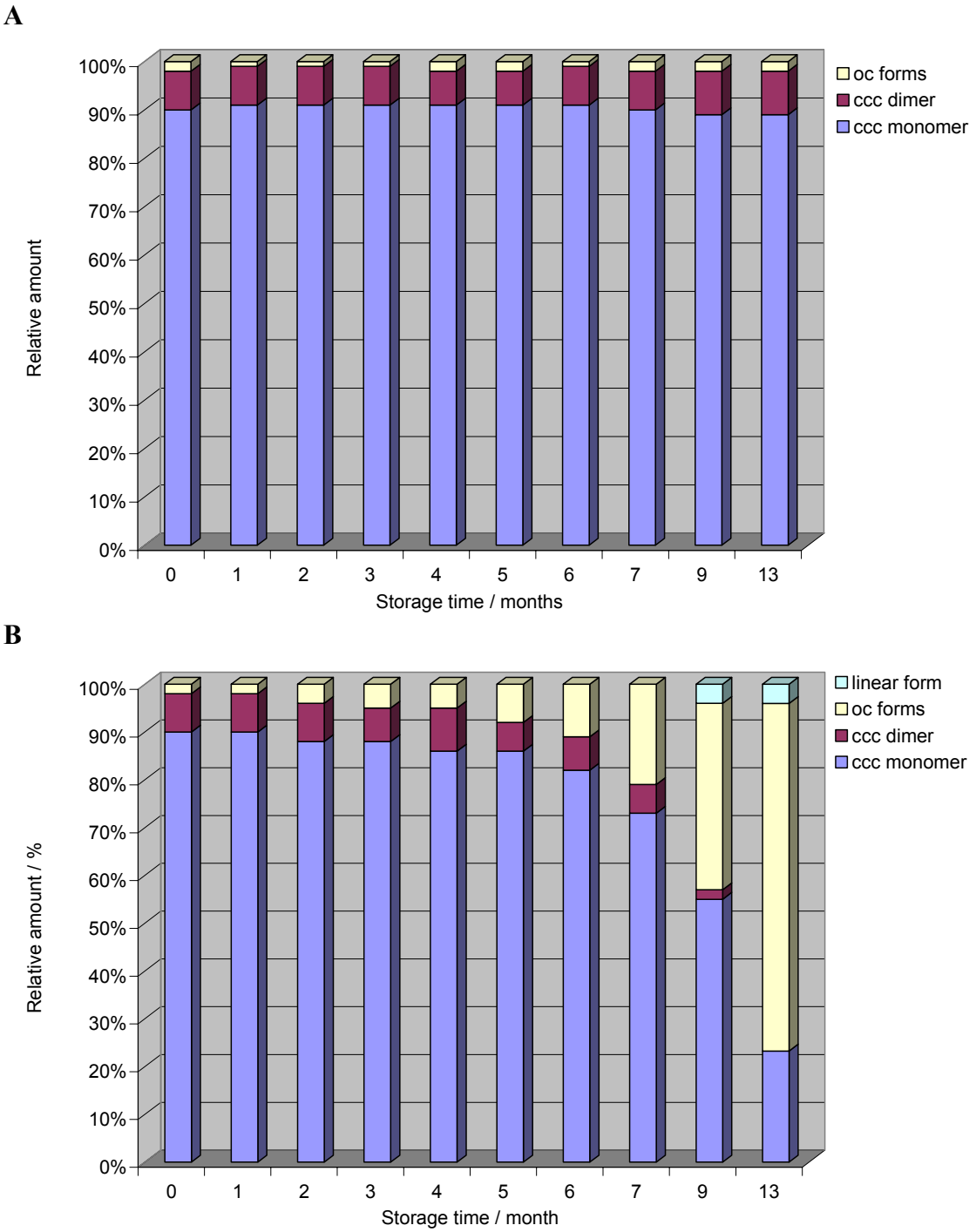


Figure 71 Long term storage stability of plasmid DNA at -80 °C (A) and 4 °C (B) (Walther *et al.*, 2003).

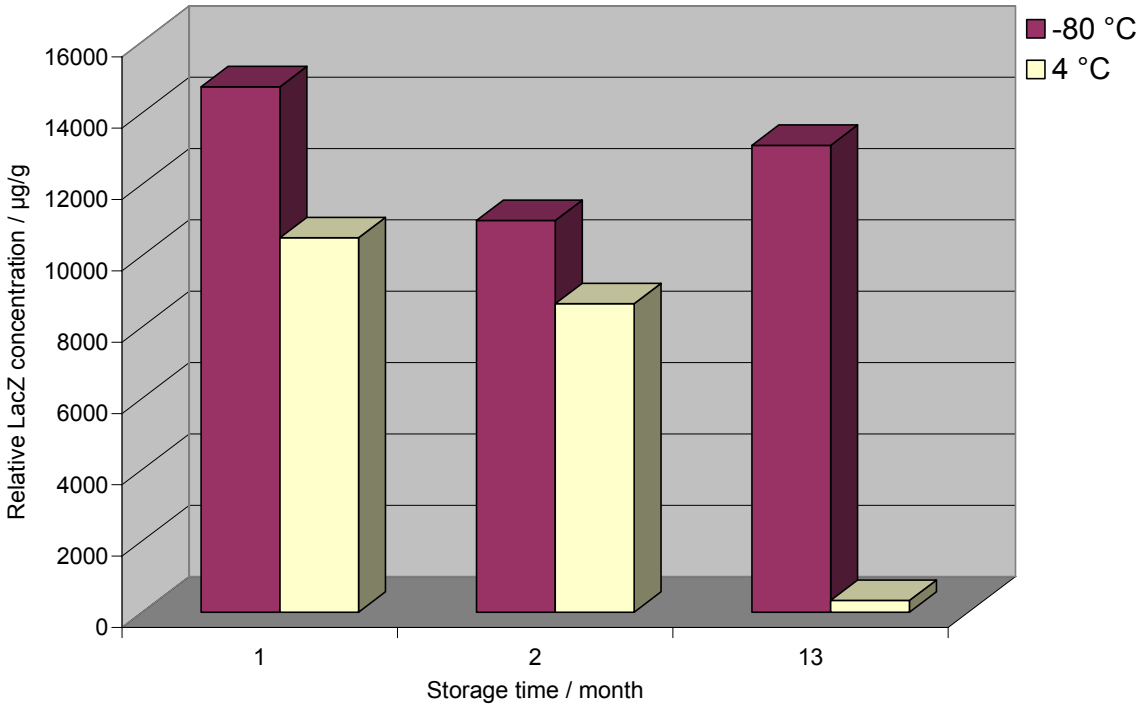


Figure 72 Expression of β-galactosidase in jet-injected xenotransplanted colon carcinomas in nude mice with plasmid DNA stored on longer term at different temperatures (Walther *et al.*, 2003).

Long term stability could be achieved by storage at -80 °C over a period of 13 month which was certainly sufficient to cover the time between production and application of a plasmid based DNA pharmaceutical.

4.6.2 Conclusions

The issue of plasmid stability has not been addressed in the literature yet. Although product stability is commonly monitored throughout the purification process, the stability during storage and application has not been of major importance. It could be shown that significant changes in plasmid DNA form distribution due to improper storage conditions had a profound effect on the efficiency of protein expression in tranfected cells. It should also be recognized that the amount of supercoiled form could not only be decreased by improper storage but also by the method used for transferring the DNA into the cells which was shown by analyzing the DNA used in jetgun injections.

5 Final Discussion, Current Research, and Future Directions

Plasmid DNA manufacturing for pharmaceutical application has proven to be a highly innovative field of research that brings up new aspects that are not only relevant for this product but have application in other areas of bioprocess engineering as well.

The cultivation of plasmid harboring *E. coli* has already been dealt with in previous work. The aim of these early studies was to develop a cultivation process that was capable of producing comparably large amounts of high quality plasmid DNA taking into account regulatory guidelines. During this development, a simple hypothesis connecting plasmid replication with specific growth rate was formulated (Schmidt, 1998; Voß, 2001; Voss *et al.*, 2004). However, this simple theory did not explain all the observations made in subsequent research that dealt with development of cultivation processes on defined media (Bär, 2005). Although a basic change in the composition of the media led to more optimized processes, still more questions came up. A more thorough analysis of these observations has been carried out by Fischer (2008) who could show the influence of the physiological state of the inoculation culture on the development of product concentration in the main bioreactor culture. Proteome analysis also gave valuable results indicating a reaction to stress and the initiation of the stringent response. Further analysis is currently directed to analyze these indications that are probably due to nutrient limitation or osmotic stress and that can explain the decreasing plasmid concentrations during late exponential growth and stationary phase. Because these stress factors also influence product formation in *Escherichia coli* in general, these experiments can also be considered as fundamental with respect to plasmid based recombinant protein expression.

Furthermore, a cultivation process has been proposed that resulted in high product selectivity with an overall high product concentration by growing an inoculation culture in semidefined HSG-medium followed by bioreactor cultivation on defined GGM- or k-GGM medium. Here, the loss of product observed in earlier experiments could be avoided giving a broader time frame for cell harvesting.

The cultivation process is followed by cell disruption. Alkaline lysis is most frequently used in plasmid purification for this purpose. The time course of this procedure and op-

timization of reaction conditions has already been analyzed by other researchers (Ciccolini *et al.*, 1998; Clemson and Kelly, 2003). Serious problems were observed when this method was performed at a larger scale as a simple batch operation. The main issue was considered to be the contamination of the product stream with small fragments of chromosomal DNA that could not be removed easily. The second problem that was postulated was insufficient lysis due to pH extremes. The separation of precipitated material from the product liquid was achieved by centrifugation and subsequent filtration. Because of the viscoelastic properties of the precipitate, this two step procedure was absolutely necessary.

Therefore, a process was developed that consisted of a continuous alkaline lysis step followed by froth flotation for clarification of the product stream. Although the latter step was not designed for a continuous mode of operation, future developments should solve this small disadvantage. In comparison to the classical method, we were able to show that an extremely clear bacterial lysate could be prepared. Furthermore, the concentration of chromosomal DNA was very low in comparison to other processes which is a serious advantage with respect to subsequent downstream processing. Another outstanding feature was the productivity of the procedure owing to a dramatic reduction of the operating time. Future research in this field should be directed to the development of a completely continuous process.

The bacterial cleared lysate was further processed to deplete the major impurities and to concentrate the product stream. For these initial downstream processing steps, several different options have been explored.

The application of a recombinant RNase was used in the style of RNase A treatment for the preparation of plasmid DNA as a cloning vector. The presented results showed that the RNase Ba from *B. amyloliquefaciens* could be produced by recombinant expression in *E. coli*. The enzyme showed an activity that was comparable to RNase A. Furthermore, no deleterious DNase activity could be observed. In comparison to state of the art methods used in pharmaceutical plasmid purification, the application of a recombinant RNase might be a serious alternative depending on its commercial availability and its economic feasability. Current research is directed to develop optimized production systems. Strong promoters as well as the application of secretory pathways (Beshay *et al.*,

2007; Choi *et al.*, 2000; Economou, 1999; Kleist *et al.*, 2003; Miksch *et al.*, 2002; Miksch *et al.*, 1997) and subsequent application of bacteriocin release proteins (BRPs) (Sommer *et al.*, 2008; van der Wal *et al.*, 1995) are applied to increase product concentration and simplify its recovery. Future research should be directed to the immobilization of the enzyme for process intensification and the integration of the immobilized RNase into different process schemes.

Affinity separations are highly selective purification techniques. Only few examples dealt with the purification of nucleic acids, especially plasmid DNA. Next to triple helix formation, protein-DNA interaction was analyzed for this purpose. Zinc finger proteins as well as the lac repressor and a few other examples have already been described in the literature. In our research, the selectivity of LacI and the replication initiator protein RepU has been analyzed by simple gel shift assays. Both proteins could be immobilized to chromatographic matrices in a non covalent manner *via* the interaction of a His_6 tag with an immobilized metal complex on the stationary phase. First results showed very poor binding capacities. This disadvantage could be overcome by the immobilization to macroporous monolithic materials. With these systems binding capacities could be achieved that were comparable or even better than already published values for such affinity interactions. However, the most serious disadvantage was the low selectivity of these systems in the chromatographic mode. Current projects try to minimize the size of the ligand and to increase its stability. Furthermore, the chromatographic parameters are optimized with respect to a more efficient separation of plasmid DNA and RNA. Future research should be carried out in the field of chemically synthesized affinity ligands for nucleic acids. The IMAC principle is utilized for protein purification and is constantly developed further by the design of more effective ligands for metal complex formation (Graham *et al.*, 2007). In plasmid purification it has only been applied for the removal of RNA. On the contrary, the DNA binding properties of metal complexes are well known from anti cancer drugs like cis-platinum (Richards and Rodger, 2007). Sophisticated ligands should be designed on this basis and tested subsequently.

The extraction of nucleic acids using reverse micellar two-phase systems has only been described with respect to the partitioning of small DNA fragments (Goto *et al.*, 2004; Goto *et al.*, 1999). In this thesis, the partitioning of RNA and plasmid DNA in a reverse micellar phase system was described. The results showed that a separation of plasmid

DNA from RNA was possible just by forward extraction or by a combined forward and back extraction. The first option could be applied to a preconditioned cleared lysate and RNA could be removed from the product stream under appropriate conditions (Streitner *et al.*, 2007). The second process option was also able to remove RNA completely and furthermore to concentrate the product stream and was, therefore, analyzed more thoroughly. Especially the back extraction was difficult to optimize because a simple change of the ionic strength in the aqueous phase did not lead to significant product recoveries. However, since the micellar structure was also influenced by the addition of alcohols as cosolvents (Kahlweit *et al.*, 1990; 1991) conditions could be identified for complete recovery of the plasmid DNA and full separation from the contaminating RNA (Streitner *et al.*, 2008). These extraction systems have been characterized further with respect to the depletion of additional contaminants, *i.e.* protein, chromosomal DNA, and endotoxins and the extraction procedure has successfully been integrated into several purification schemes which were capable to produce high quality plasmid DNA that met the given specifications.

With respect to the mechanism of the extraction process, all results indicated a separation by size according to the principles that are generally accepted for this process. If the plasmid DNA is accomodated by clusters of reverse micelles or by a micellar network structure, is currently analyzed. This research should also give valuable results for the interpretation of the separation mechanism. In the future, extraction processes have the capacity to work as an initial purification step in many other processes. The high product concentrations in monoclonal antibody production make highly scalable and selective procedures necessary. Here, reverse micellar extraction or aqueous two-phase systems might be an attractive alternative. A comprehensive analysis of the physical properties of these systems with a focus on the separation process and chemical synthesis of tailor made surfactants should be one goal in the future. Furthermore, the selectivity of the extraction process might be enhanced by using affinity tagged surfactants. In this way, extraction can be coupled with the design of affinity ligands which will result in a close connection of both research themes.

The stability of plasmid DNA was determined during storage and application. Analysis of plasmid DNA subjected to different injection pressures using jet gun injection and long term storage at different temperatures was achieved with capillary gel electropho-

resis. In the first case, conditions could be identified that were considered as an optimal balance between sufficient transfection efficiency and minimal degradation of supercoiled DNA. The effect of low amounts of ccc form on the expression of reporter proteins in xenotransplanted carcinoma cells was shown in the second case. A significant reduction of the reporter protein expression could be observed after prolonged storage at 4 °C which coincided with a considerable decrease of the ccc amount.

Because CGE is very sensitive to product contaminations, especially proteins, and about 50 minutes are necessary for analysis, faster methods that are less sensitive to protein contamination should be developed. Current research focuses on the development of surface modified capillaries and matrices for capillary electrochromatography (CEC). By introducing less hydrophobic ligands and matrices, protein binding should be minimized. Furthermore, the electrophorectic flow profile should result in reasonably good separation in a short time.

As overall summary, plasmid DNA can be produced by sophisticated batch operation using defined media. *E. coli* can be disrupted by alkaline lysis in a semi continuous process combined with froth flotation for clarification. For subsequent downstream processing, a reverse micellar extraction process has been developed that is capable of sufficient removal of chromosomal DNA, proteins, and LPS when integrated into a sophisticated process scheme. Digestion by RNase Ba can be used as an alternative for RNA removal. However, the additional contaminations have to be depleted by other purification techniques. DNA-protein interaction did not yet meet the expectations set in an affinity technique. Further research in the field of plasmid DNA affinity purification is advisable. The same applies for the assessment of plasmid DNA stability. Although current techniques are available for this purpose, more sophisticated ones have to be developed. Additionally, further research should be directed to the mechanism of DNA degradation in solution. Here, appropriate methods for the analysis of degradation products have to be established as well.

In addition to the topics presented in this thesis, further research is conducted in the design of chromatographic matrices for plasmid purification. The diploma thesis of Philipp Claar (Claar, 2007) started research in this area by comparing grafted and ungrafted monolithic columns for protein and plasmid purification.

In conclusion, pharmaceutical plasmid DNA manufacturing remains challenging and the adventure continues.

Ancora Imparo – Yet I am learning

Michelangelo

6 Appendix

6.1 Abbreviations and Symbols

α	selectivity
μ	specific growth rate
μ_{max}	maximum specific growth rate
ϕ_E	degree of extraction
a	enzyme activity
A	absorption
AA	amino acid
ADA	adenosine desaminase
ADP	adenosine diphosphate
AGE	agarose gel electrophoresis
AIX	anion exchange chromatography
AML	affinity macro ligand
ATCase	aspartate transcarbamoylase
ATP	adenosing triphosphate
ATPS	aqueous two-phase system
AOT	aerosol OT, sodium bisethylhexyl sulfosuccinate
B.Sc.	bachelor of science
ccc	closed covalently circular
CD	circular dichroism
c_E	solute concentration in extract
CGE	capillary gel electrophoresis

chr.	chromosomal
CIM	convective interaction media
c_l	actual oxygen concentration
c_l^*	oxygen equilibrium concentration
c_R	solute concentration in raffinate
CTP	cytidine triphosphate
CV	column volume
Da	Dalton
DHAP	dihydroxyacetone phosphate
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
DO	dissolved oxygen
EMEA	European medicines agency
FDA	Food and Drug Administration (USA)
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthase
GMP	good manufacturing practice
GMP	guanosine monophosphate
HIC	hydrophobic interaction chromatography
His_6	hexahistidine
HIV	human immune defficiency virus
IDA	iminodiacetic acid
IMP	inosinic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside

K	partition coefficient
kb	kilo base pairs
k_La	volumetric oxygen transport coefficient
Lac	Lactose
LacI	Lac inhibitor
LacZ	β-galactosidase
LIF	laser induced fluorescence
LPS	lipopolysaccharides, endotoxins
MALDI	matrix assisted laser desorption/ionization
mRNA	messenger RNA
n/a	not available
n.d.	not detectable
NIPAM	N-isopropyl acrylamide
oc	open circular
OD	optical density
OTR	oxygen transfer rate
OUR	oxygen utilization rate
P	plasmid DNA concentration
PAGE	polyacrylamide gel electrophoresis
pDNA	plasmid DNA
PEG	polyethylene glycol
PRPP	phosphoribosyl pyrophosphate
RepU	replication initiator protein U from plasmid pUB110
RNA	ribonucleic acid
RNase	ribonuclease

r_P	product formation rate
r_S	substrate utilization rate
r_X	growth rate
SDS	sodium dodecylsulfate
SEC	size exclusion chromatography
siRNA	small interfering RNA
$S_{P/X}$	product selectivity, biomass specific product yield coefficient, represents average plasmid copy number
t	time
TOF	time of flight
TOMAC	methyltrioctyl ammonium chloride
U	units, enzymatic activity
UTP	uridine triphosphate
VI	vinyl imidazole
x	fraction
X	dry biomass concentrations
$Y_{P/S}$	substrate specific product yield coefficient
$Y_{X/S}$	substrate specific biomass yield coefficient

6.2 Media Composition

Table 25 HSG-medium (Voß *et al.*, 2003).

Compound	Concentration / g L^{-1}
Glycerol	14.9
Soybean peptone	13.5
Yeast extract	7
K_2HPO_4	2.3
KH_2PO_4	1.5
NaCl	2.5
$MgSO_4 \cdot 7\ H_2O$	0.25

Table 26 Composition of TB medium.

Compound	Concentration / g L^{-1}
Glycerol	5
Tryptone	12
Yeast extract	24
K_2HPO_4	12.5
KH_2PO_4	2.3

Table 27 GGM-medium (Bär, 2005).

Compound	Concentration / g L^{-1}
Glycerol	14.9
KH_2PO_4	1.5
K_2HPO_4	2.3
NaCl	2.5
$MgSO_4 \cdot 7\ H_2O$	0.25
Citric acid $\cdot\ H_2O$	5.0
$(NH_4)_2SO_4$	2.5
Thiamine $\cdot$ HCl	0.010
Trace element solution	2 mL L^{-1}

Table 28 Trace element solution (Voss *et al.*, 2004).

Compound	Concentration / g L^{-1}
$FeCl_3 \cdot 6\ H_2O$	5.4
$ZnSO_4 \cdot 7\ H_2O$	1.38
$MnSO_4 \cdot H_2O$	1.85
$CoSO_4 \cdot 7\ H_2O$	0.56
$CuCl_2$	0.17
Boric acid	1.0
$NaMoO_4 \cdot 2\ H_2O$	2.5
Citric acid $\cdot\ H_2O$	5.0

Table 29 Composition of GGM and k-GGM (Fischer, 2008).

Compound	GGM / g L^{-1}	k-GGM / g L^{-1}
Glycerol	14.9	29.8
KH_2PO_4	1.5	3.0
K_2HPO_4	2.3	4.6
NaCl	2.5	2.5
Na_3citrate · 5.5 H_2O	9.5	9.5
$MgSO_4$ · 7 H_2O	0.25	0.75
$(NH_4)_2SO_4$	2.5	10.0
Trace element solution	2 mL L^{-1}	2 mL L^{-1}
Thiamine · HCl	0.0112	0.0168

Table 30 Composition of HSG(+) applied in Voß (2001).

Compound	HSG(+) / g L^{-1}
Glycerol	17.4
Yeast extract	10
Soybean peptone	10
NaCl	2.5
K_2HPO_4	2.3
KH_2PO_4	1.5
$MgSO_4$ · 7 H_2O	0.25
Na-L-glutamate	15

Table 31 Composition of defined media applied in Voß (2001).

Compound	Glycerol glutamate medium / g L^{-1}	M9-medium / g L^{-1}
Glycerol	52.2	-
Glucose · H_2O	-	11.0
Na-L-Glutamat	30	-
Na_2HPO_4 x 7 H_2O	-	1.0
KH_2PO_4	1.5	3.0
K_2HPO_4	2.3	-
NaCl	2.5	0.5
$MgSO_4$ x 7 H_2O	1	-
Thiamine · HCl	10 mg L^{-1}	-
Trace element solution	2 mL L^{-1}	-
$(NH_4)_2SO_4$	-	2.49
$ZnSO_4$ · 7 H_2O	-	1.4 mg L^{-1}
$FeCl_3$ · 6 H_2O	-	5.4 mg L^{-1}
$MnSO_4$	-	1.6 mg L^{-1}
$CuCl_2$	-	0.17 mg L^{-1}
$CoSO_4$ · 7 H_2O	-	0.562 mg L^{-1}
$MgCl_2$ · 7 H_2O	-	0.221
$CaCl_2$	-	14.7 mg L^{-1}

6.3 Supervised Theses

6.3.1 PhD thesis

Streitner, Nadine	„Extraktion von Nukleinsäuren mittels inversmizellarer Zweiphasensysteme (Extraction of nucleic acids using reverse micellar two-phase systems)“ Since 07/2005

6.3.2 Diploma theses

Schulze-Horsel, Josef	„Anwendung der Nitratatmung in anaeroben Fermentationsprozessen (Application of nitrate respiration in anaerobic cultivation processes)“ 10/2002 – 06/2003
Kessler, Lars Christian	„Untersuchung und Charakterisierung von Phytinsäure in ihrer Funktion als niedermolekularer Verdränger von Plasmid-DNA (Examination and characterization of phytic acid as a low molecular weight displacer of plasmid DNA)“ 01/2004 – 09/2004
Lindau, Dennis	„Herstellung einer rekombinanten Ribonuklease Ba und Einsatz zur Aufarbeitung von pharmazeutischer Plasmid-DNA (Production of recombinant Ribonuclease Ba and its use in downstream processing of pharmaceutical plasmid DNA)“ 01/2004 – 09/2004

Hasche, Anja	„Aufreinigung und Immobilisierung des *lac*-Repressors aus *E. coli* zur affinitätsbasierten Isolierung biopharmazeutisch einsetzbarer Plasmide (Purification and immobilization of the *lac* repressor from *E. coli* for affinity based purification of biopharmaceutical plasmid DNA)“
	03/2004 – 12/2004
Kortmann, Hendrik	„Eignung von Rep-Proteinen zur affinitätsbasierten Isolierung biopharmazeutisch einsetzbarer Plasmide am Beispiel des RepU (Suitability of Rep proteins for affinity based isolation of biopharmaceutical plasmid DNA exemplified by RepU)“
	05/2004 – 02/2005
Bär, Antonia	„Supplementierung eines synthetischen Minimalmediums mit Aminosäuren zur Steigerung der Plasmid-Produktion in *E. coli* (Supplementation of a synthetic minimal medium with amino acids for increased plasmid production in *E. coli*)“
	06/2004 – 03/2005
Huang, Xiaohong	„Verwendung des Barnase-Barstar-Systems zur Affinitätsaufarbeitung rekombinanter Proteine (Use of the barnase-barstar-system for affinity purification of recombinant proteins)“
	07/2004 – 05/2005
Kellner, Alexander	„Extraktion von Nukleinsäuren als pharmazeutischer Wirkstoff mittels inversmizellarer Zweiphasensysteme (Extraction of nucleic acids as pharmaceutical drugs using reverse micellar two-phase systems)“
	01/2005 – 09/2005

Block, Martin	„Rekombinante Herstellung des Replikationsinitiatorproteins A (RepA) als Affinitätsligand für Plasmid-DNA (Recombinant production of the replication initiator protein A (RepA) as affinity ligand for plasmid DNA)“ 02/2005 – 10/2005
Husemann, Ilka	„Entwicklung von robotikgestützten Screeningverfahren für die Prozessentwicklung in der Proteinreinigung (Development of robitic supported screening platforms for process development in protein purification)“ (external thesis) 07/2005 – 03/2006
Abel, Jürgen	„Fusionsproteine aus GFP_{UV} und RepU an monolithischen Trägermaterialien zur Aufarbeitung von Plasmid-DNA für biopharmazeutische Zwecke (Fusion proteine of GFP_{UV} and RepU on monolithic supports for pharmaceutical plasmid DNA puification)“ 03/2006 – 12/2006
Schrewe, Manfred	„Optimierung der Herstellung des DNA-bindenden Proteins RepU (Optimizing the production of the DNA-binding protein RepU)“ 03/2006 – 12/2006
Claar, Phillip	„Synthese monolithischer Festphasen für den Labormaßstab (Synthesis of monolithic matrices at the laboratory scale)“ 02/2007 – 11/2007

Bellwied, Petra	"Immobilisierung von DNA-bindenden Proteinen und Untersuchung der nukleinsäurebindenden Eigenschaften (Immobilization of DNA-binding proteins and examination of nucleic acid binding properties)"
	05/2007 – 01/2008
Fischer, Jan	"Untersuchung der Plasmidreplikation in *E. coli* bei Kultivierung in synthetischen und halbsynthetischen Medien sowie Analyse des Proteoms (Examination of plasmid replication in E. coli during cultivations on defined and semidefined media as well as proteome analysis) "
	04/2007 – 01/2008

6.3.3 B.Sc. thesis

Kirsten Tschapalda	"Isolierung von Plasmid-DNA durch inversmizellare Zweiphasensysteme - Analytik der zellulären Verunreinigungen (Isolation of plasmid DNA by reverse micellar two-phase systems – analysis of cellular contaminations)"

7 Bibliography

Abel, J, 2006. Fusionsproteine aus GFPuv und RepU an monolithischen Trägermaterialien zur Aufarbeitung von Plasmid-DNA für biopharmazeutische Zwecke. *Diploma Thesis*, Bielefeld University, Faculty of Technology.

Albertsson, P A, 1965. Partition Studies on Nucleic Acids. I. Influence of Electrolytes, Polymer Concentration and Nucleic Acid Conformation of the Partition in the Dextran-Polyethylene Glycol System. *Biochim Biophys Acta* **103:** 1-12.

Albertsson, P A, 1986. Partitioning of cell particles and macromolecules. J. Wiley & Sons, New York.

Arvidsson, P, Plieva, F M, Savina, I N, Lozinsky, V I, Fexby, S, Bulow, L, Galaev, I Y and Mattiasson, B, 2002. Chromatography of microbial cells using continuous supermacroporous affinity and ion-exchange columns. *J Chromatogr A* **977:** 27-38.

Atlung, T, Christensen, B B and Hansen, F G, 1999. Role of the rom protein in copy number control of plasmid pBR322 at different growth rates in Escherichia coli K-12. *Plasmid* **41:** 110-9.

Azevedo, A M, Rosa, P A, Ferreira, I F and Aires-Barros, M R, 2007. Optimisation of aqueous two-phase extraction of human antibodies. *J Biotechnol* **132:** 209-17.

Bailey, J E and Ollis, D F, 1986. Biochemical engineering fundamentals. McGraw-Hill, New York.

Balan, S, Murphy, J, Galaev, I, Kumar, A, Fox, G E, Mattiasson, B and Willson, R C, 2003. Metal chelate affinity precipitation of RNA and purification of plasmid DNA. *Biotechnol Lett* **25:** 1111-6.

Bär, A, 2005. Supplementierung eines synthetischen Minimalmediums mit Aminosäuren zur Steigerung der Plasmid-Produktion in *E. coli*. *Diploma Thesis*, Bielefeld University, Faculty of Technology.

Bellwied, P, 2008. Immobilisierung von DNA-bindenden Proteinen und Untersuchung der nukleinsäurebindenden Eigenschaften. *Diploma Thesis*, Bielefeld University, Faculty of Technology.

Beshay, U, Miksch, G, Friehs, K and Flaschel, E, 2007. Increasing the secretion ability of the kil gene for recombinant proteins in Escherichia coli by using a strong stationary-phase promoter. *Biotechnol Lett* **29:** 1893-901.

Birnboim, H C and Doly, J, 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7:** 1513-23.

Bolivar, F, 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique Eco RI sites for selection of Eco RI generated recombinant DNA molecules. *Gene* **4:** 121-36.

Borek, E, Rockenbach, J and Ryan, A, 1956. Studies on a mutant of Escherichia coli with unbalanced ribonucleic acid synthesis. *J Bacteriol* **71:** 318-23.

Boyer, J D, Cohen, A D, Vogt, S, Schumann, K, Nath, B, Ahn, L, Lacy, K, Bagarazzi, M L, Higgins, T J, Baine, Y, Ciccarelli, R B, Ginsberg, R S, MacGregor, R R and Weiner, D B, 2000. Vaccination of Seronegative Volunteers with a Human Immunodeficiency Virus Type 1 env/rev DNA Vaccine Induces Antigen Specific Proliferation and Lymphocyte Production of Chemokines. *J Infect Dis* **181:** 476-483.

Cano, T, Murphy, J C, Fox, G E and Willson, R C, 2005. Separation of genomic DNA from plasmid DNA by selective renaturation with immobilized metal affinity capture. *Biotechnol Prog* **21:** 1472-7.

Carlson, A, Signs, M, Liermann, L, Boor, R and Jim Jem, K, 1995. Mechanical disruption of *Escherichia coli* for plasmid recovery. *Biotechnol Bioeng* **48:** 303-315.

Carnes, A E, Hodgson, C P and Williams, J A, 2006. Inducible Escherichia coli fermentation for increased plasmid DNA production. *Biotechnol Appl Biochem* **45:** 155-66.

Cashel, M, Gentry, D R, Hernandez, V J and Vinella, D, 1996. The stringent response. In: F. C. Neidhardt, Escherichia coli and Salmonella Cellular and molecular biology, ASM Press, Washington.

Chang, D-E, Smalley, D J and Conway, T, 2002. Gene expression profiling of Escherichia coli growth transitions: an expanded stringent response model. *Mol Microbiol* **45:** 289-306.

Check, E, 2002. A tragic setback. *Nature* **420:** 116-8.

Chen, W, Graham, C and Ciccarelli, R B, 1997. Automated fed-batch fermentation with feed-back controls based on dissolved oxygen (DO) and pH for production of DNA vaccines. *J Ind Microbiol Biotechnol* **18:** 43-8.

Chen, Z Y, He, C Y, Ehrhardt, A and Kay, M A, 2003. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol Ther* **8:** 495-500.

Chen, Z Y, He, C Y and Kay, M A, 2005. Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression in vivo. *Hum Gene Ther* **16:** 126-31.

Choi, J H, Jeong, K J, Kim, S C and Lee, S Y, 2000. Efficient secretory production of alkaline phosphatase by high cell density culture of recombinant Escherichia coli using the Bacillus sp. endoxylanase signal sequence. *Appl Microbiol Biotechnol* **53:** 640-5.

Ciccolini, L A, Shamlou, P A, Titchener-Hooker, N J, Ward, J M and Dunnill, P, 1998. Time course of SDS-alkaline lysis of recombinant bacterial cells for plasmid release. *Biotechnol Bioeng* **60:** 768-70.

Cichutek, K, 2001. Gentherapie: Gut' Ding will Weile haben. In: P.-E.-I. Langen, Editorial, Bundesgesundheitsblatt Gesundheitsforschung-Gesundheitsschutz, Springer-Verlag, Berlin, Heidelberg.

Claar, P, 2007. Synthese monolithischer Festphasen für den Labormaßstab. *Diploma Thesis*, Bielefeld University, Faculty of Technology.

Clemson, M and Kelly, W J, 2003. Optimizing alkaline lysis for DNA plasmid recovery. *Biotechnol Appl Biochem* **37:** 235-44.

Costioli, M D, Fisch, I, Garret-Flaudy, F, Hilbrig, F and Freitag, R, 2003. DNA purification by triple-helix affinity precipitation. *Biotechnol Bioeng* **81:** 535-45.

Dainiak, M B, Plieva, F M, Galaev, I Y, Hatti-Kaul, R and Mattiasson, B, 2005. Cell chromatography: separation of different microbial cells using IMAC supermacroporous monolithic columns. *Biotechnol Prog* **21:** 644-9.

D'Alessio, G, Di Donato, A, Mazzarella, L and Piccoli, R, 1997. Seminal ribonuclease: the importance of diversity. In: G. D'Alessio and J. F. Riordan, Ribonucleases: Structures and functions, Academic Press, New York.

Danko, I and Wolff, J A, 1994. Direct gene transfer into muscle. *Vaccine* **12:** 1499-502.

Diogo, M M, Queiroz, J A and Prazeres, D M, 2001a. Studies on the retention of plasmid DNA and Escherichia coli nucleic acids by hydrophobic interaction chromatography. *Bioseparation* **10:** 211-20.

Diogo, M M, Ribeiro, S C, Queiroz, J A, Monteiro, G A, Tordo, N, Perrin, P and Prazeres, D M, 2001b. Production, purification and analysis of an experimental DNA vaccine against rabies. *J Gene Med* **3:** 577-84.

Duarte, S P, Fortes, A G, Prazeres, D M and Marcos, J C, 2007. Preparation of plasmid DNA polyplexes from alkaline lysates by a two-step aqueous two-phase extraction process. *J Chromatogr A* **1164:** 105-12.

Duenas-Carrera, S, Vina, A, Martinez, R, Alvarez-Lajonchere, L, Alvarez-Obregon, J C, Marante, J, Perez, A, Mosqueda, O, Martinez, G and Morales, J, 2004. Immunization with a DNA vaccine encoding the hepatitis-C-virus structural antigens elicits a specific immune response against the capsid and envelope proteins in rabbits and Macaca irus (crab-eating macaque monkeys). *Biotechnol Appl Biochem* **39:** 249-55.

Economou, A, 1999. Following the leader: bacterial protein export through the Sec pathway. *Trends Microbiol* **7:** 315-20.

EMEA, 2001. Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products. *CPMP/410/01 rev 1, London*

Eon-Duval, A and Burke, G, 2004. Purification of pharmaceutical-grade plasmid DNA by anion-exchange chromatography in an RNase-free process. *J Chromatogr B* **804:** 327-35.

FDA, 1998. Guidance for industry: Guidance for human somatic cell therapy and gene therapy. *Center for Biologics Evaluation and Research, FDA, Rockville, MD*

Ferreira, G N, Cabral, J M and Prazeres, D M, 1998. Purification of supercoiled plasmid DNA using chromatographic processes. *J Mol Recognit* **11:** 250-1.

Ferreira, G N, Cabral, J M and Prazeres, D M, 2000. Anion exchange purification of plasmid DNA using expanded bed adsorption. *Bioseparation* **9:** 1-6.

Fexby, S, Nilsson, A, Hambraeus, G, Tjerneld, F and Bulow, L, 2004. Partitioning and characterization of tyrosine-tagged green fluorescent proteins in aqueous two-phase systems. *Biotechnol Prog* **20:** 793-8.

Fischer, J, 2008. Untersuchung der Plasmidreplikation in *E. coli* bei Kultivierung in synthetischen und halbsynthetischen Medien sowie Analyse des Proteoms. *Diploma Thesis*, Bielefeld University, Faculty of Technology.

Fishman, D M and Patterson, G D, 1996. Light scattering studies of supercoiled and nicked DNA. *Biopolymers* **38:** 535-52.

Forde, G M, Ghose, S, Slater, N K, Hine, A V, Darby, R A and Hitchcock, A G, 2006. LacO-LacI interaction in affinity adsorption of plasmid DNA. *Biotechnol Bioeng* **95:** 67-75.

Frerix, A, Geilenkirchen, P, Muller, M, Kula, M R and Hubbuch, J, 2007. Separation of genomic DNA, RNA, and open circular plasmid DNA from supercoiled plasmid DNA by combining denaturation, selective renaturation and aqueous two-phase extraction. *Biotechnol Bioeng* **96:** 57-66.

Frerix, A, Muller, M, Kula, M R and Hubbuch, J, 2005. Scalable recovery of plasmid DNA based on aqueous two-phase separation. *Biotechnol Appl Biochem* **42:** 57-66.

Fu, J Y, Potty, A S, Fox, G E and Willson, R C, 2006. Water-elutability of nucleic acids from metal-chelate affinity adsorbents: enhancement by control of surface charge density. *J Mol Recognit* **19:** 348-53.

Galaev, I and Mattiasson, B, 1993. Thermoreactive water-soluble polymers, nonionic surfactants, and hydrogels as reagents in biotechnology. *Enzyme Microb Technol* **15:** 354-66.

Ghose, S, Forde, G M and Slater, N K, 2004. Affinity adsorption of plasmid DNA. *Biotechnol Prog* **20:** 841-50.

Göklen, K E and Hatton, T A, 1987. Liquid-liquid extraction of low molecular-weight proteins by selective solubilization in reversed micelles. *Sep Sci Technol* **22:** 831-841.

Goto, M, Momota, A and Tsutomu, O, 2004. DNA extraction by cationic reverse micelles. *J Chem Eng Jpn* **37:** 662-668.

Goto, M, Ono, T, Horiuchi, A and Furusaki, S, 1999. Extraction of DNA by reversed micelles. *J Chem Eng Jpn* **32:** 123-125.

Graham, B, Comba, P, Hearn, M T and Spiccia, L, 2007. An examination of the binding behavior of histidine-containing peptides with immobilized metal complexes derived from the macrocyclic ligand, 1,4,7-triazacyclononane. *J Biol Inorg Chem* **12:** 11-21.

Gu, Z and Glatz, C E, 2007. Aqueous two-phase extraction for protein recovery from corn extracts. *J Chromatogr B Analyt Technol Biomed Life Sci* **845:** 38-50.

Gustavsson, P E, Lemmens, R, Nyhammar, T, Busson, P and Larsson, P O, 2004. Purification of plasmid DNA with a new type of anion-exchange beads having a non-charged surface. *J Chromatogr A* **1038:** 131-40.

Hahn, R, Bauerhansl, P, Shimahara, K, Wizniewski, C, Tscheliessnig, A and Jungbauer, A, 2005. Comparison of protein A affinity sorbents II. Mass transfer properties. *J Chromatogr A* **1093:** 98-110.

Hahnen, E, Znamenskaya, L, Koczan, D, Leshchinskaya, I and Hobom, G, 2000. A novel secreted ribonuclease from Bacillus intermedius: gene structure and regulatory control. *Mol Gen Genet* **263:** 571-80.

Hanora, A, Savina, I, Plieva, F M, Izumrudov, V A, Mattiasson, B and Galaev, I Y, 2006. Direct capture of plasmid DNA from non-clarified bacterial lysate using polycation-grafted monoliths. *J Biotechnol* **123:** 343-55.

Harman, J G, 2001. Allosteric regulation of the cAMP receptor protein. *Biochim Biophys Acta* **1547:** 1-17.

Hartley, R W, 1989. Barnase and barstar: two small proteins to fold and fit together. *Trends Biochem Sci* **14:** 450-4.

Hartley, R W and Rogerson, D L, Jr., 1972. Production and purification of the extracellular ribonuclease of Bacillus amyloliquefaciens (barnase) and its intracellular inhibitor (barstar). I. Barnase. *Prep Biochem* **2:** 229-42.

Hasche, A, 2004. Aufreinigung und Immobilisierung des lac-Repressors aus *E. coli* zur affinitätsbasierten Isolierung biopharmazeutisch einsetzbarer Plasmide. *Diploma Thesis*, Bielefeld University, Faculty of Technology.

Hasche, A and Voß, C, 2005. Immobilisation of a repressor protein for binding of plasmid DNA. *J Chromatogr A* **1080:** 76-82.

Hatton, T A, 1989. Reversed micellar extraction of proteins. In: J. F. Scamehorn and J. H. Harwell, Surfactant based separation processes, Marcel Dekker Inc., New York, Basel.

He, L and Niemeyer, B, 2003. A novel correlation for protein diffusion coefficients based on molecular weight and radius of gyration. *Biotechnol Prog* **19:** 544-8.

Hebel, H, Attra, H, Khan, A and Draghia-Akli, R, 2006. Successful parallel development and integration of a plasmid-based biologic, container/closure system and electrokinetic delivery device. *Vaccine* **24:** 4607-14.

Helmann, J D, 1991. Alternative sigma factors and the regulation of flagellar gene expression. *Mol Microbiol* **5:** 2875-2882.

Hentsch, M, Menoud, P, Steiner, L, Flaschel, E and Renken, A, 1992. Optimization of the surfactant (AOT) concentration in a reverse micellar extraction process. *Biotechnol Tech* **6:** 359-364.

Hiller, J, Franco-Lara, E and Weuster-Botz, D, 2007. Metabolic profiling of Escherichia coli cultivations: evaluation of extraction and metabolite analysis procedures. *Biotechnol Lett* **29:** 1169-78.

Holmes, D S and Quigley, M, 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal Biochem* **114:** 193-7.

Hong, D-P and Kuboi, R, 1999. Evaluation of the alcohol-mediated interaction between micelles using percolation processes of reverse micellar systems. *Biochem Eng J* **4:** 23-29.

Horn, U, Strittmatter, W, Krebber, A, KnÃ¼pfer, U, Kujau, M, Wenderoth, R, MÃ¼ller, K, Matzku, S, PlÃ¼ckthun, A and Riesenberg, D, 1996. High volumetric yields of functional dimeric miniantibodies in *Escherichia coli*, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions. *Appl Microbiol Biotechnol* **46:** 524-532.

Huang, S, Ratliff, K S, Schwartz, M P, Spenner, J M and Matouschek, A, 1999. Mitochondria unfold precursor proteins by unraveling them from their N-termini. *Nat Struct Biol* **6:** 1132-8.

Ilyina, T V and Koonin, E V, 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. *Nucleic Acids Res* **20:** 3279-85.

Imre, V E and Luisi, P L, 1982. Solubilization and condensed packaging of nucleic acids in reversed micelles. *Biochem Biophys Res Commun* **107:** 538-45.

Ito, T, Smith, C L and Cantor, C R, 1992. Sequence-specific DNA purification by triplex affinity capture. *Proc Natl Acad Sci U S A* **89:** 495-8.

Johansson, H O, Magaldi, F M, Feitosa, E and Pessoa, A, Jr., 2008. Protein partitioning in poly(ethylene glycol)/sodium polyacrylate aqueous two-phase systems. *J Chromatogr A* **1178:** 145-53.

Joyce, A R, Reed, J L, White, A, Edwards, R, Osterman, A, Baba, T, Mori, H, Lesely, S A, Palsson, B O and Agarwalla, S, 2006. Experimental and computational assessment of conditionally essential genes in Escherichia coli. *J Bacteriol* **188:** 8259-71.

Kahlweit, M, Strey, R and Busse, G, 1990. Microemulsions: a qualitative thermodynamic approach. *J Phys Chem* **94:** 3881-3894.

Kahlweit, M, Strey, R and Busse, G, 1991. Effect of alcohols on the phase behavior of microemulsions. *J Phys Chem* **95:** 5344-5352.

Kepka, C, Lemmens, R, Vasi, J, Nyhammar, T and Gustavsson, P E, 2004a. Integrated process for purification of plasmid DNA using aqueous two-phase systems combined with membrane filtration and lid bead chromatography. *J Chromatogr A* **1057:** 115-24.

Kepka, C, Rhodin, J, Lemmens, R, Tjerneld, F and Gustavsson, P E, 2004b. Extraction of plasmid DNA from Escherichia coli cell lysate in a thermoseparating aqueous two-phase system. *J Chromatogr A* **1024:** 95-104.

Kleist, S, Miksch, G, Hitzmann, B, Arndt, M, Friehs, K and Flaschel, E, 2003. Optimization of the extracellular production of a bacterial phytase with Escherichia coli by using different fed-batch fermentation strategies. *Appl Microbiol Biotechnol* **61:** 456-62.

Kortmann, H, 2005. Eignung von Rep-Proteinen zur affinitätsbasierten Isolierung biopharmazeutisch einsetzbarer Plasmide, am Beispiel des RepU. *Diploma Thesis*, Bielefeld University, Faculty of Technology.

Kramberger, P, Petrovic, N, Strancar, A and Ravnikar, M, 2004. Concentration of plant viruses using monolithic chromatographic supports. *J Virol Methods* **120:** 51-7.

Kula, M R, 1990. Trends and future prospects of aqueous two-phase extraction. *Bioseparation* **1:** 181-9.

Kumar, A, Galaev, I and Mattiasson, B, 1999. Purification of Lac repressor protein using polymer displacement and immobilization of the protein. *Bioseparation* **8:** 307-16.

Kumar, P K, Maschke, H E, Friehs, K and Schügerl, K, 1991. Strategies for improving plasmid stability in genetically modified bacteria in bioreactors. *Trends Biotechnol* **9:** 279-84.

Kusano, K, Nakayama, K and Nakayama, H, 1989. Plasmid-mediated lethality and plasmid multimer formation in an Escherichia coli recBC sbcBC mutant. Involvement of RecF recombination pathway genes. *J Mol Biol* **209:** 623-34.

Lahijani, R, Hulley, G, Soriano, G, Horn, N A and Marquet, M, 1996. High-yield production of pBR322-derived plasmids intended for human gene therapy by employing a temperature-controllable point mutation. *Hum Gene Ther* **7:** 1971-80.

Lee, L P and Tidor, B, 2001. Barstar is electrostatically optimized for tight binding to barnase. *Nat Struct Biol* **8:** 73-6.

Lee, S Y, 1996. High cell-density culture of *Escherichia coli*. *Trends Biotechnol* **14:** 98-105.

Lemmens, R, Olsson, U, Nyhammar, T and Stadler, J, 2003. Supercoiled plasmid DNA: selective purification by thiophilic/aromatic adsorption. *J Chromatogr B* **784:** 291-300.

Leser, M E, Wei, G, Luisi, P L and Maestro, M, 1986. Application of reverse micelles for the extraction of proteins. *Biochem Biophys Res Commun* **135:** 629-635.

Levy, M S, O'Kennedy, R D, Ayazi-Shamlou, P and Dunnill, P, 2000. Biochemical engineering approaches to the challenges of producing pure plasmid DNA. *Trends Biotechnol* **18:** 296-305.

Li, Y and Beitle, R R, 2002. Protein purification via aqueous two-phase extraction (ATPE) and immobilized metal affinity chromatography. Effectiveness of salt addition to enhance selectivity and yield of GFPuv. *Biotechnol Prog* **18:** 1054-9.

Lin, E C C, 1996. Dissimilation pathways for sugars, polyols, and carboxylates. In: F. C. Neidhardt, Escherichia coli and Salmonella Cellular and molecular biology, ASM Press, Washington.

Lin-Chao, S, Chen, W T and Wong, T T, 1992. High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II. *Mol Microbiol* **6:** 3385-93.

Lindau, D, 2004. Herstellung einer rekombinanten Ribonuklease Ba und Einsatz zur Aufarbeitung von pharmazeutischer Plasmid-DNA. *Diploma Thesis*, Bielefeld University, Faculty of Technology.

Ljunglöf, A, Bergvall, P, Bhikhabhai, R and Hjorth, R, 1999. Direct visualisation of plasmid DNA in individual chromatography adsorbent particles by confocal scanning laser microscopy. *J Chromatogr A* **844:** 129-35.

Loewen, P C and Hengge-Aronis, R, 1994. The role of the sigma factor sigma S (KatF) in bacterial global regulation. *Annu Rev Microbiol* **48:** 53-80.

Lucht, J M and Bremer, E, 1994. Adaptation of Escherichia coli to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system proU. *FEMS Microbiol Rev* **14:** 3-20.

Lundeberg, J, Wahlberg, J and Uhlen, M, 1990. Affinity purification of specific DNA fragments using a lac repressor fusion protein. *Genet Anal Tech Appl* **7:** 47-52.

Märkl, H, Zenneck, C, Dubach, A C and Ogbonna, J C, 1993. Cultivation of *Escherichia coli* to high cell densities in a dialysis reactor. *Appl Microbiol Biotechnol* **39:** 48-52.

Matejtschuk, P, 1997. Affinity separations. IRL Press, Oxford.

Middelberg, A P J, 1995. Process-scale disruption of microorganisms. *Biotechnol Adv* **13:** 491-551.

Miksch, G, Kleist, S, Friehs, K and Flaschel, E, 2002. Overexpression of the phytase from Escherichia coli and its extracellular production in bioreactors. *Appl Microbiol Biotechnol* **59:** 685-94.

Miksch, G, Neitzel, R, Fiedler, E, Friehs, K and Flaschel, E, 1997. Extracellular production of a hybrid beta-glucanase from Bacillus by Escherichia coli under different cultivation conditions in shaking cultures and bioreactors. *Appl Microbiol Biotechnol* **47:** 120-6.

Mossakowska, D E, Nyberg, K and Fersht, A R, 1989. Kinetic characterization of the recombinant ribonuclease from Bacillus amyloliquefaciens (barnase) and investigation of key residues in catalysis by site-directed mutagenesis. *Biochemistry* **28:** 3843-50.

Murphy, J C, Jewell, D L, White, K I, Fox, G E and Willson, R C, 2003. Nucleic acid separations utilizing immobilized metal affinity chromatography. *Biotechnol Prog* **19:** 982-6.

Mutzall, K, 1993. Einführung in die Fermentationstechnik. Behr's Verlag, Hamburg.

Neidhardt, F C, 1996. Escherichia coli and Salmonella. Cellular and molecular biology.

Nielsen, J and Villadsen, J, 1994. Bioreaction engineering principles. Plenum Press, New York, London.

Ohlsson, R, Hentschel, C C and Williams, J G, 1978. A rapid method for the isolation of circular DNA using an aqueous two-phase partition system. *Nucleic Acids Res* **5:** 583-90.

O'Kennedy, R D, Baldwin, C and Keshavarz-Moore, E, 2000. Effects of growth medium selection on plasmid DNA production and initial processing steps. *Journal of Biotechnology* **76:** 175-183.

O'Kennedy, R D and Patching, J W, 1997. Effects of medium composition and nutrient limitation on loss of the recombinant plasmid pLG669-z and beta-galactosidase expression by Saccharomyces cerevisiae. *J Ind Microbiol Biotechnol* **18:** 319-25.

Okorokov, A L, Hartley, R W and Panov, K I, 1994. An improved system for ribonuclease Ba expression. *Protein Expr Purif* **5:** 547-52.

O'Mahony, K, Freitag, R, Hilbrig, F, Muller, P and Schumacher, I, 2005. Proposal for a better integration of bacterial lysis into the production of plasmid DNA at large scale. *J Biotechnol* **119:** 118-32.

O'Mahony, K, Freitag, R, Hilbrig, F, Schumacher, I and Muller, P, 2007. Integration of bacteria capture via filtration and in situ lysis for recovery of plasmid DNA under industry-compatible conditions. *Biotechnol Prog* **23:** 895-903.

Pastan, I and Perlman, R, 1970. Cyclic Adenosine Monophosphate in Bacteria: In many bacteria the synthesis of inducible enzymes requires this cyclic nucleotide. *Science* **169:** 339-344.

Peterka, M, Jarc, M, Banjac, M, Frankovic, V, Bencina, K, Merhar, M, Gaberc-Porekar, V, Menart, V, Strancar, A and Podgornik, A, 2006. Characterisation of metal-chelate methacrylate monoliths. *J Chromatogr A* **1109:** 80-5.

Pietrini, A V and Luisi, P L, 2002. Circular dichroic properties and average dimensions of DNA-containing reverse micellar aggregates. *Biochim Biophys Acta* **1562:** 57-62.

Podgornik, A, Barut, M, Strancar, A, Josic, D and Koloini, T, 2000. Construction of large-volume monolithic columns. *Anal Chem* **72:** 5693-9.

Poppenborg, L and Flaschel, E, 1994. Affinity extraction of proteins by means of reverse micellar phases containing a metal-chelating surfactant. *Biotechnol Tech* **8:** 307-312.

Poppenborg, L, Friehs, K and Flaschel, E, 1997. The green fluorescent protein is a versatile reporter for bioprocess monitoring. *J Biotechnol* **58:** 79-88.

Porath, J, 1992. Immobilized metal ion affinity chromatography. *Protein Expr Purif* **3:** 263-81.

Potty, A S, Fu, J Y, Balan, S, Haymore, B L, Hill, D J, Fox, G E and Willson, R C, 2006. Neutral additives enhance the metal-chelate affinity adsorption of nucleic acids: role of water activity. *J Chromatogr A* **1115:** 88-92.

Powell, K, 2004. DNA vaccines--back in the saddle again? *Nat Biotechnol* **22:** 799-801.

Ramachandran, S and Udgaonkar, J B, 1996. Stabilization of barstar by chemical modification of the buried cysteines. *Biochemistry* **35:** 8776-85.

Reinikainen, P, Korpela, K, Nissinen, V, Olkku, J, Söderlund, H and Markkanen, P, 1989. *Escherichia coli* plasmid production in fermenter. *Biotechnol Bioeng* **33:** 386-393.

Ribeiro, S C, Monteiro, G A, Cabral, J M and Prazeres, D M, 2002. Isolation of plasmid DNA from cell lysates by aqueous two-phase systems. *Biotechnol Bioeng* **78:** 376-84.

Richards, A D and Rodger, A, 2007. Synthetic metallomolecules as agents for the control of DNA structure. *Chem Soc Rev* **36:** 471-83.

Riesenberg, D and Guthke, R, 1999. High-cell-density cultivation of microorganisms. *Appl Microbiol Biotechnol* **51:** 422-430.

Rushizky, G W, Greco, A E, Hartley, R W, Jr. and Sober, H A, 1964. Studies on the Characterization of Ribonucleases. *J Biol Chem* **239:** 2165-9.

Schluep, T and Cooney, C L, 1998. Purification of plasmids by triplex affinity interaction. *Nucleic Acids Res* **26:** 4524-8.

Schmidt, M, Babu, K R, Khanna, N, Marten, S and Rinas, U, 1999a. Temperature-induced production of recombinant human insulin in high-cell density cultures of recombinant Escherichia coli. *J Biotechnol* **68:** 71-83.

Schmidt, T, 1998. Produktion und Analytik von Plasmid-DNA für die Gentherapie. *PhD Thesis*, Bielefeld University, Faculty of Technology.

Schmidt, T, Friehs, K, Schleef, M, Voss, C and Flaschel, E, 1999b. Quantitative analysis of plasmid forms by agarose and capillary gel electrophoresis. *Anal Biochem* **274:** 235-40.

Schmidt, T, Schleef, M, Friehs, K and Flaschel, E, 1999c. Hochzelldichtefermentation zur Gewinnung von Plasmid-DNA für Gentherapie und genetische Impfung. *BioForum* **22:**

Schorr, J, Moritz, P, Seddon, T and Schleef, M, 1995. Plasmid DNA for human gene therapy and DNA vaccines. Production and quality assurance. *Ann N Y Acad Sci* **772:** 271-3.

Schroeckh, V, Hartmann, M, Birch-Hirschfeld, E and Riesenberg, D, 1992. Improvement of recombinant gene expression in Escherichia coli for glucose-controlled continuous and fed-batch cultures. *Appl Microbiol Biotechnol* **36:** 487-492.

Schulze-Horsel, J, 2003. Anwendung der Nitratatmung in anaeroben Fermentationsprozessen. *Diploma Thesis*, Bielefeld University, Faculty of Technology.

Selber, K, Nellen, F, Steffen, B, Thommes, J and Kula, M R, 2000. Investigation of mathematical methods for efficient optimisation of aqueous two-phase extraction. *J Chromatogr B Biomed Sci Appl* **743:** 21-30.

Sommer, B, Schmidt, B, Friehs, K and Flaschel, E, 2008. Extrazelluläre Produktion und Affinitätsreinigung einer rekombinanten Ribonuklease mit Escherichia coli. *Chem Ing Tech* **80:** 839-845.

Stadler, J, Lemmens, R and Nyhammar, T, 2004. Plasmid DNA purification. *J Gene Med* **6 Suppl 1:** S54-66.

Stolberg, S G, 1999. The biotech death of Jesse Gelsinger. *N Y Times Mag* 136-140, 149-150.

Strancar, A, Podgornik, A, Barut, M and Necina, R, 2002. Short monolithic columns as stationary phases for biochromatography. *Adv Biochem Eng Biotechnol* **76:** 49-85.

Streitner, N, Voß, C and Flaschel, E, 2007. Reverse micellar extraction systems for the purification of pharmaceutical grade plasmid DNA. *J Biotechnol* **131:** 188-196.

Streitner, N, Voß, C and Flaschel, E, 2008. Isolierung von Plasmid-DNA durch inversmizellare Zweiphasensysteme - Optimierung der Rückextraktion. *Chem Ing Tech* **80:** 831-837.

Summers, D K, 1996. The biology of plasmids. Blackwell Science, Cambridge USA.

Svec, F and Huber, C G, 2006. Monolithic materials: Promises, challenges, achievements. *Anal Chem* **78:** 2101-7.

Sweet, G, Gandor, C, Voegele, R, Wittekindt, N, Beuerle, J, Truniger, V, Lin, E C and Boos, W, 1990. Glycerol facilitator of Escherichia coli: cloning of glpF and identification of the glpF product. *J Bacteriol* **172:** 424-30.

Thömmes, J, Halfar, M, Gieren, H, Curvers, S, Takors, R, Brunschier, R and Kula, M R, 2001. Human chymotrypsinogen B production from Pichia pastoris by integrated development of fermentation and downstream processing. Part 2. Protein recovery. *Biotechnol Prog* **17:** 503-12.

Trindade, I P, Diogo, M M, Prazeres, D M and Marcos, J C, 2005. Purification of plasmid DNA vectors by aqueous two-phase extraction and hydrophobic interaction chromatography. *J Chromatogr A* **1082:** 176-84.

Tschapalda, K, 2008. Isolierung von Plasmid-DNA durch inversmizellare Zweiphasensysteme - Analytik der zellulären Verunreinigungen. *B.Sc. Thesis*, Bielefeld University, Faculty of Technology.

Twigg, A J and Sherratt, D, 1980. Trans-complementable copy-number mutants of plasmid ColE1. *Nature* **283:** 216-8.

Tyn, M T and Gusek, T W, 1990. Prediction of diffusion coefficients of proteins. *Biotechnol Bioeng* **35:** 327-338.

Urthaler, J, Ascher, C, Wohrer, H and Necina, R, 2007. Automated alkaline lysis for industrial scale cGMP production of pharmaceutical grade plasmid-DNA. *J Biotechnol* **128:** 132-49.

Urthaler, J, Schlegl, R, Podgornik, A, Strancar, A, Jungbauer, A and Necina, R, 2005. Application of monoliths for plasmid DNA purification development and transfer to production. *J Chromatogr A* **1065:** 93-106.

van der Wal, F J, Luirink, J and Oudega, B, 1995. Bacteriocin release proteins: mode of action, structure, and biotechnological application. *FEMS Microbiol Rev* **17:** 381-99.

Viklund, C, Svec, F, Frechet, J M and Irgum, K, 1997. Fast ion-exchange HPLC of proteins using porous poly(glycidyl methacrylate-co-ethylene dimethacrylate) monoliths grafted with poly(2-acrylamido-2-methyl-1-propanesulfonic acid). *Biotechnol Prog* **13:** 597-600.

Visca, P, Leoni, L, Wilson, M J and Lamont, I L, 2002. Iron transport and regulation, cell signalling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. *Mol Microbiol* **45:** 1177-1190.

Voß, C, 2001. Neue Verfahren zur Herstellung von Plasmid-DNA als Biopharmazeutikum. *PhD Thesis*, Bielefeld University, Faculty of Technology.

Voß, C, 2008. Aufarbeitung von Plasmid-DNA für Gentherapie und genetische Impfung. *Chem Ing Tech* **80:** 795-802.

Voss, C, 2007. Production of plasmid DNA for pharmaceutical use. *Biotechnol Annu Rev* **13C:** 201-222.

Voss, C, Lindau, D and Flaschel, E, 2006. Production of recombinant RNase Ba and its application in downstream processing of plasmid DNA for pharmaceutical use. *Biotechnol Prog* **22:** 737-44.

Voss, C, Schmidt, T and Schleef, M, 2005. From bulk to delivery: Plasmid manufacturing and storage. In: M. Schleef, DNA-Pharmaceuticals, Wiley-VCH, Weinheim, Germany.

Voß, C, Schmidt, T, Schleef, M, Friehs, K and Flaschel, E, 2003. Production of supercoiled multimeric plasmid DNA for biopharmaceutical application. *J Biotechnol* **105:** 205-213.

Voss, C, Schmidt, T, Schleef, M, Friehs, K and Flaschel, E, 2003. Production of supercoiled multimeric plasmid DNA for biopharmaceutical application. *J Biotechnol* **105:** 205-213.

Voss, C, Schmidt, T, Schleef, M, Friehs, K and Flaschel, E, 2004. Effect of ammonium chloride on plasmid DNA production in high cell density batch culture for biopharmaceutical use. *J Chem Technol Biotechnol* **79:** 57-62.

Walther, W, Stein, U, Fichtner, I, Voss, C, Schmidt, T, Schleef, M, Nellessen, T and Schlag, P M, 2002. Intratumoral low-volume jet-injection for efficient nonviral gene transfer. *Mol Biotechnol* **21:** 105-15.

Walther, W, Stein, U, Voss, C, Schmidt, T, Schleef, M and Schlag, P M, 2003. Stability analysis for long-term storage of naked DNA: impact on nonviral in vivo gene transfer. *Anal Biochem* **318:** 230-5.

Wan, N C, McNeilly, D S and Christopher, C W, 1998. Method for lysing cells., US 5 837 529.

Wang, F and Lee, S Y, 1998. High cell density culture of metabolically engineered Escherichia coli for the production of poly(3-hydroxybutyrate) in a defined medium. *Biotechnol Bioeng* **58:** 325-8.

Weuster-Botz, D, 2000. Experimental design for fermentation media development: statistical design or global random search? *J Biosci Bioeng* **90:** 473-83.

Weuster-Botz, D, Puskeiler, R, Kusterer, A, Kaufmann, K, John, G T and Arnold, M, 2005. Methods and milliliter scale devices for high-throughput bioprocess design. *Bioprocess Biosyst Eng* **28:** 109-19.

Wick, L M and Egli, T, 2004. Molecular components of physiological stress responses in Escherichia coli. *Adv Biochem Eng Biotechnol* **89:** 1-45.

Wolff, J A, Ludtke, J J, Acsadi, G, Williams, P and Jani, A, 1992. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum Mol Genet* **1:** 363-9.

Wolff, J A, Malone, R W, Williams, P, Chong, W, Acsadi, G, Jani, A and Felgner, P L, 1990. Direct gene transfer into mouse muscle in vivo. *Science* **247:** 1465-8.

Woll, J M, Hatton, T A and Yarmush, M L, 1989. Bioaffinity separations using reversed micellar extraction. *Biotechnol Bioeng* **5:** 57-62.

Woodgate, J, Palfrey, D, Nagel, D A, Hine, A V and Slater, N K, 2002. Protein-mediated isolation of plasmid DNA by a zinc finger-glutathione S-transferase affinity linker. *Biotechnol Bioeng* **79:** 450-6.

Xie, S, Allington, R W, Frechet, J M and Svec, F, 2002. Porous polymer monoliths: an alternative to classical beads. *Adv Biochem Eng Biotechnol* **76:** 87-125.