1 Introduction and aims of the thesis

Starting at the very latest with the development of the polymerase chain reaction (Mullis et al., 1986), the development and production of more complex biological products has begun to increase exponentially (Carlson, 2016). Biological products, ranging from simple organic compounds up to highly complex recombinant designer proteins, span into almost every region of our daily life and industrial demands. Hereby, the range has changed from the biotechnological production of already existing products (e.g. insulin, lipases) to rationally designed chimeric proteins which combine the properties of several completely different sources. The latter ones can be used for example as medicine for various types of cancer, vaccination and several other diseases (Glick et al., 2010) and will in the near future conclude in a more personalized medicine. The development and production of these bioproducts often prove tedious, time consuming and expensive. Finally, at the end of each production process, the products have to be purified to separate them from contaminants and pollutants in order to guarantee a safe and high quality product. To this end, the *current good manufacturing practice* (CGMP) has been implemented as mandatory for pharmaceutical products (European Commission - Public Health, 2011; U.S. Department of Health and Human Services, 1997). The CGMP enforces a very strict logging of data, ensuring a safe and reproducible process. It provides guidelines concerning strength, identity, quality and purity of the product. To this end, a very high degree of process control and automation from the obtaining of raw materials to the sale of the final formulated drug has to be established and recorded. This includes continuous testing and validation of established and registered processes. The term *current* in CGMP refers to the fact that processes have to use up to date-technologies. With research progressing, the thresholds for product quality change. To take this development into account, the guidelines are changing in time.

The efficiencies and cost of separation and purification change dramatically, depending on the origin of the process. In chemical processes, very high purities can be reached in highly reproducible set-ups. However, products of low molecular weights are often combined with stereo-selective centers. Even more complex products, i.e. proteins, cannot readily be produced in chemical processes. The introduction of the necessary protection groups for stereo- and region-selective reactions combined with high reaction times, for the chemical processes, shifts the

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cost effectiveness of these processes towards a purely biological application. Recent medical advances are mostly based on these more complex molecules and even products with a very high molecular mass and complicated structure, which would not be possible to produce using chemical processes. Here, the substrates are processed by complex, biological materials. In biological processes, the catalysts can be either single enzymes or, more often, whole cells. Sometimes, the product is the cell itself (Anupama and Ravindra, 2000; ICI, 1976). Biological processes however often have to deal with very low productivities compared with chemical reactions. The handling of living and constantly growing cells often proves challenging. On the other hand, very complex products can often be produced in a very low number of process steps and sometimes during a single experimental set-up. Therefore, the outcome can be very rewarding. These biological systems using whole cells are further divided into bacterial, fungal, mammalian and insect cell cultures, depending on the origin of the producing organism. Further classification is done using the strain and biological family of the organism itself.

In the production of pharmaceutical products, the final purity of the product is crucial, as the application for patients cannot possibly be done using impure or even contaminated products. Therefore, the product separation and purification are a very important stages within the whole process. These steps are called *downstream processing* (DSP). Strain selection, cell banking and cultivation until the start of the harvest are called *upstream processing*. Both processes are either up or down of the process, i.e. stream. In recent years, research acknowledged that the upstream processing and DSP can and should be improved individually at first. But as parts of the process are tightly intertwined, changes on one part can lead to hugely different conditions on the other end of the process chain. For example, changing parts of the growth medium or the whole cultivation medium can result in higher product titers, but may however reduce the overall yield, as the purification becomes more complex due to impurities inherit in the new parts of the medium or unspecific binding of those to the purification material. Obviously, the connections become very complex while changing single parameters. Also, the development and optimization of technical protein biosynthesis and DSP cannot be *a priori* predictive due to lack of knowledge. These steps are carried out iteratively with partial variation of individual process steps within each cycle. Thus, developments in process optimization are time consuming and cost intensive but can lead to the economic feasibility of new processes and

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therefore higher quality and safety for the consumer. Up to now, a holistic approach to the development and biotechnological production of effective biopharmaceuticals incorporating interdisciplinary use of biochemistry, systems biology and process engineering has not been established (Gädke et al., 2017b).

The separation and purification of products from the biotechnological production process itself have always been non-trivial. This is shown by the very high number of purification protocols both in lab and industrial scale and is evident in the cost of product purification, which can range up to 80 % of the overall process costs for pharmaceutical products (Gagnon, 2012; Roque et al., 2004). The separation itself is always based on a different interaction of the product with the separation material in contrast to the pollutants. Therefore, step by step, different pollutants can be separated using different separation methods. However, as each separation step has an efficiency < 100 %, a higher number of steps is correlated to a higher loss of product. An exemplary process yield based on the number of steps is shown in **Figure 1**. While a very high yield of 95 % per step results in a total yield of 60 % after 10 separate purification steps, a yield of 75 % results in an overall yield of 6 % after the same number of steps. An increased necessity of purification steps to achieve a given purity threshold results in higher loss of product in the course of the purification.

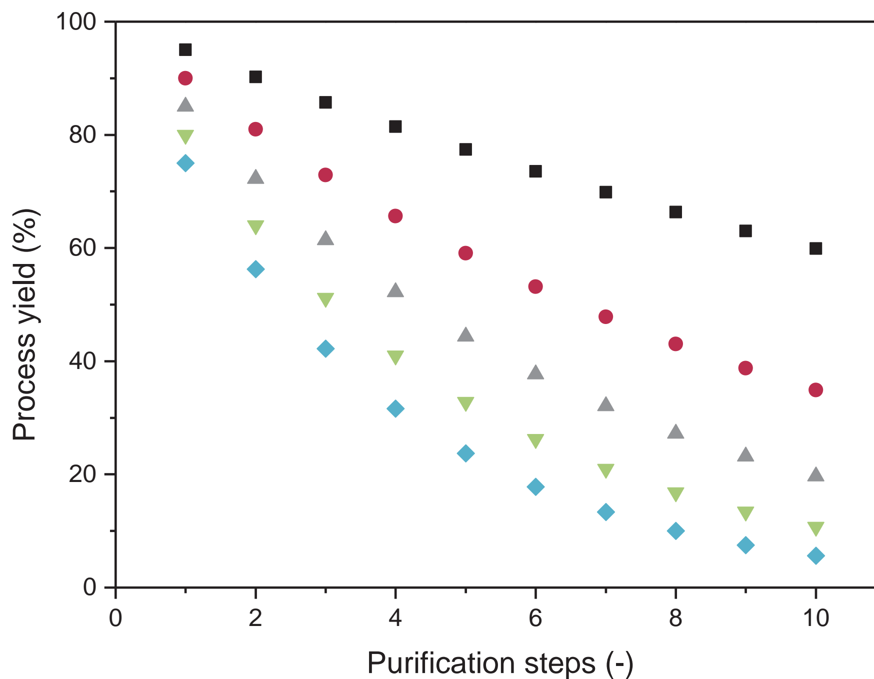

***Figure 1: Process yield based on yield per purification step and number of purification steps** (decreasing from top to bottom symbols: 95 % (black squares), 90 % (red dots), 85 % (grey triangles), 80 % (green triangles), 75 % (blue diamonds) yield per step).*

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A mere screening for a reduced number of purification steps often results in less efficient processes, as the combination of different steps into one is not trivial. However, the overall cost might be reduced as every purification step needs specialized equipment and staff for its professional and economical operation. Efficient DSP development therefore means searching for completely new or improved processes and optimizing and adapting the given process conditions in conjunction with the upstream processing. Sophisticated examples from biotechnological industry and research are integrated purification processes where the addition of separating agents into the fermentation broth efficiently removes the product. This often results in less unwanted inhibitions through product-feedback.

Usually, the first purification step is the separation of the biomass from the surrounding cultivation medium. Depending on whether the product is intra- or extracellular, the cells are discarded or further processed. For intracellular processes, the cells have to be disrupted. This entails the separation of intracellular metabolites and can also include unfinished product. Furthermore, most cell disrupting processes are prone to damaging the product either because of very high (or low) temperatures or other extreme process conditions (Schütte and Kula, 2001). Furthermore, disrupted cells cannot be used for further production. In the case of extracellular products, the culture supernatant is most times reduced in volume and then applied to a chromatographic separation unit. In this unit, the product binds to the fixed matrix of the chromatography column due to binding affinities. Afterwards, the product is eluted from the column material and further purified using ion exchange and crystallization processes. These steps are often repeated in several iterations and/or under different conditions until the product reaches the desired final purity (Gronemeyer et al., 2014).

This scientific work focusses on a downstream process. The aims of the present thesis were:

- Two different model products of importance for the biotechnological and pharmaceutical industry, Protein A and α-Lysozyme D1.3scFv antibody fragment, should be used in the course of this thesis.
- The production of the two model proteins entailed the development of a vector construct enabling for high secretion rates of recombinant extracellular proteins combined with evaluation in *Bacillus megaterium* the host model

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organism. This research was conducted in cooperation with the Institute of Microbiology (ifM), TU Braunschweig (AG *Biedendieck*).

- The development of an integrated product separation process using super paramagnetic iron oxide nanoparticles (SPION). These particles were developed and functionalized with chemical compounds to elicit affinity towards a histidine tag and provided by the cooperation partner of the Institute of Particle Technology (AG *Garnweitner/Kleinfeldt*), TU Braunschweig. The two provided particle systems had to be thoroughly analyzed and furthermore compared with a commercially available particle system.
- The different SPION-based particle systems had to be analyzed on their usability for *in situ* affinity chromatography of recombinant extracellular products.
- The purification protocol has to incorporate possible particle regeneration and reusability in subsequent cycles. These regenerated particles had to be analyzed and balanced in five consecutive growth experiments.
- In each purification step, the process was to be investigated towards its performance in terms of product purity, adsorption and elution of the product.
- Finally, leakage of the chelation-mediating agent Ni^{2+} into the different fractions of the process had to be evaluated.
- Additionally, the separation should be performable using handheld neodymium magnets. This method potentially allows for the combination of several DSP steps, namely solid-liquid separation, affinity chromatography and buffer exchange, into one integrated step of the cultivation.
- This separation had to be implemented in shake flasks and a lab-scale bioreactor. In the latter, the process should be performed automatically to increase its robustness.
- The biological process itself, using *Bacillus megaterium* as host cell for production, had to be analyzed and improved based on the secretion of the recombinant Protein A and cellular growth behavior.

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2 Current state of research

2.1 Bacterial production systems

Production of (high value) products in biological systems is highly depending on the organism chosen for their biosynthesis. Often, the product could be produced in different systems. However, advantages and disadvantages of each system have to be considered while selecting the optimal organism. Nowadays, the production systems are divided in the following categories: a) Mammalia cell cultures, which are mostly used for proteins which need posttranslational modifications (Glick et al., 2010). A well-known representative here is the Chinese hamster ovary (CHO) cell line from the hamster *Cricetulus griseus*, insect cell lines, which are mostly used for the amplification of viruses, mostly Baculoviridae, b) fungal cell lines, which are used for the production of lipases or drinking ethanol, for which *Pichia pastoris* and *Saccharomyces* sp. are well-known, c) plant cell systems for the production of vaccines, e.g. in *Arabidopsis thaliana* or tobacco plants (Glick et al., 2010), and d) Archaea which were thought to live only in extreme habits, but are also common in moderate environments are seldom used (Bang and Schmitz, 2015). The last group of production systems includes bacteria, which can be mostly divided into *Gram*-negative and –positive bacteria. But there are also *Gram*-variable and –undefined bacteria (Steinberg and Burd, 2005). In the following, proteins will be the focus of this work. Other metabolites will not be referred to, if not stated otherwise.

Bacteria are not able to post-translationally modify proteins, i.e. modification of proteins after their synthesis in the process of the mRNA translation. They are missing the compartmentalization and respective modification mechanisms which are present in eukaryotes (Butler and Spearman, 2014). However, they combine very high product titers with high specific growth rates and moderate medium requirements. They are highly resistant to mechanical stress, especially *Gram*-positive bacteria (Fuchs et al., 2007), and can therefore easily be separated using centrifugation steps (Gronemeyer et al., 2014; Weuster-Botz et al., 2007). The most obvious difference between *Gram*-positive and - negative bacteria is the structure of their cell walls. *Gram*-negative bacteria possess an inner and outer cell membrane, divided by a cell wall. The outer cell membrane consists of a thin layer of peptidoglycan. This layer can weigh up to 10 % of the dry cell membrane weight. *Gram*-positive bacteria have only one cell membrane, which has a very thick layer of

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