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DIPLOMARBEIT

RECOVERY OF PERIPLASMIC PROTEINS FROM VIABLE *E. COLI*: IMPROVE PRODUCT RELEASE IN SITU WITHOUT CELL DEATH AND LYSIS

AUSGEFÜHRT ZUM ZWECKE DER ERLANGUNG DES
AKADEMISCHEN GRADES EINES DIPLOMINGENEURS
UNTER DER LEITUNG VON

PRIVATDOZ. DR. OLIVER SPADIUT

E166, INSTITUT FÜR VERFAHRENSTECHNIK UND TECHN.
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Christoph Slouka: *Recovery of periplasmic proteins from viable E. coli: Improve product release in situ without cell death and lysis* Wien, am 11.5.2015

"Es ist ein Jammer, dass die Dummköpfe so selbstsicher sind und die Klugen so voller Zweifel"

— Bertrand Arthur William Russell

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ABSTRACT

The Gram negative bacterium *E. coli* is used in a wide range of biotechnological applications, as a result of straight forward cloning strategies and easy available cultivation techniques. Recombinant proteins are often expressed as inclusion bodies (IB), which are agglomerations of mostly misfolded protein, or as soluble cytoplasmic products, which can also be translocated to the periplasm or even the extracellular space. While recovery of extracellular proteins is rather simple, intracellular proteins and inclusion bodies in particular, demand high complex down-stream processes, often involving very cost-intensive refolding and chromatographic steps. Strategies to reduce these downstream operations, for example genetic modifications in the recombinant target protein to change the transport to different compartments within the cell or reduce and even prevent a high abundance of the formation of IB, are of high interest. Beside those genetic modifications, strategies in controlling process parameters during the fermentation process came recently into the focus of research. Different feeding strategies using mixed feeds, temperature and dO_2 control can often be used to strongly influence the bacterial cell in production of the target protein.

In this study we investigated the release of a periplasmic protein - alkaline phosophatase (AP) - to the supernatant during a fermentation process using a mixed feed strategy. As a first step the viability of the cell had to be monitored during the process. Avoiding complete cell lysis is of high importance to prevent product degradation of even product loss during the cultivation. Associated lysis monitoring was performed at- line via flow cytometric analysis using different dyes for differentiating between dead and living cells. In the course of this study a new method for online measurement of viable cell concentration using electrochemical impedance spectroscopy was tested, and applied during batch and fedbatch phases. With the possibility to determine the viable cell concentration (VCC) at-line and online, different outer membrane permeabilizing agents in combination with heat shock experiments were used to increase the release of the periplasmic protein into the supernatant. Permeability of the cells was investigated using photometric assays for alkaline phosphatase as a periplasmic protein.

ZUSAMMENFASSUNG

Das Gram negative Bakterium *E. coli* ist heutzutage einer der wichtigsten Produzenten für rekombinante Proteine, nicht nur, da etablierte Klonierungsstrategien vorhanden sind, sondern auch aufgrund der einfachen Kultivierbarkeit der Bakterien. Rekombinant hergestellte Proteine werden in *E. coli* häufig als Einschlusskörper (Inclusion bodies-IB) expressiert. Während die Aufarbeitung von gelösten und richtig gefaltenen Proteinen, im Cytoplasma oder Periplasma, als einfacher gilt, ist der Aufwand für das Prozessieren von IBs grösser. Faltungsschritte und mehrere chromatographische Aufarbeitungsschritte werden hier im Regelfall benötigt. Daher gilt Translokation ins Periplasma über Signalpeptide als günstig, da die oxidierenden Bedingungen und die Anwesenheit von Chaperonen die richtige Faltung der Proteine begünstigen. Neben genetischen Modifikationen um die Bildung von IBs zu reduzieren, wird heute wieder vermehrt Aufmerksamkeit auf die Verwendung von Prozessparametern gelegt. Alternative Fütterungsstrategien sowie Reduktion in Temperatur und Gelöstsauerstoffkontrolle sind nur ein paar Methoden um die Ausbeuten von löslichem Zielprotein zu verändern.

Diese Arbeit behandelt Möglichkeiten für den Aufschluss der äusseren Zellmembran um periplasmatische Produkte zu gewinnen ohne die Zelle zu töten und anschliessend zu desintegrieren, da Zelldesintegration im Regelfall zu Produktdegradation oder sogar -zerstörung führen kann. Als erster Schritt musste die Zellviabilität im Reaktor untersucht werden. Dazu wurde Durchflusszytometrie als at line Methode verwendet um zwischen lebenden und toten Zellen zu unterscheiden. Im Zuge dieser Untersuchungen wurde eine Messsonde auf Basis von Elektrochemischer Impedanz Spektroskopie entwickelt, die es möglich macht Lebendzellkonzentration online im Reaktor zu messen. Mit diesen Möglichkeiten konnte der Aufschluss untersucht werden. Hitzeschocks wurden durchgeführt und Chemikalien zugestzt um die äussere Membran zu permeablisieren. Die Produktausschleusung wurde mittels photometrischen Methoden über das periplasmatische Protein Alkalische Phosphatase (AP) bestimmt.

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1 INTRODUCTION

E. coli is one of the most widely used expression hosts for production of recombinant proteins as it can be cultivated on cheap minimal media to high cell densities, straightforward cloning procedures are available and high productivities can be achieved [1, 2, 3, 4, 5]. As *E. coli* does not excrete proteins, recombinant products are usually located inside the cell [6]. For product recovery cells have to be harvested, resuspended and lysed to obtain the recombinant product. Complete cell lysis leads not only to release of the product but also to release of unwanted impurities such as DNA, host cell proteins and proteases [7]. For certain applications proteins need to be exported to the periplasmic space of *E. coli*, which allows formation of disulfide bonds due to its oxidative environment. Subsequent selective disruption of the periplasm by osmotic shock after harvesting the cells leads to much purer crude extracts compared to complete cell lysis as only 4-8 % of *E. coli* host cell proteins are located in the periplasmic space [8]. However, most of the studies lack information about the integrity of the cells and do not differentiate between complete cell lysis and selective periplasmic release. Furthermore, in most cases cells were harvested from the broth, before they were resuspended in the respective lysis agent. Osmotic shock is also a commonly used method for extracting periplasmic protein from *E.coli* with high yields [7]. Generally sample preparation is cumbersome consisting of different steps: harvesting, washing, equilibrating in hyperonic solution, resuspending in distilled water. Only a few studies on releasing the periplasmic proteins directly to the cultivation broth are available to date [9, 10, 11]. Such a strategy is advantageous, since product can be obtained during cultivation and cell harvesting can be omitted. Furthermore, this approach might be promising in continuous bioprocessing as cells only get leaky but are still viable. Product is continuously harvested and cultivation can be prolonged, as lysis is kept at a minimum. Therefore, the knowledge of the viable cell concentration (VCC) is of utmost importance. In general, the viable cell concentration during cultivations is evaluated using offline measurement principles including marker proteins or fluorescence probes. Online and inline approaches are rather scarce and are based on physical measurement principles. One principle generally applied is high frequent alternating current (AC) impedance spectroscopy with high field amplitudes used on the basis of the so called β-dispersion [12, 13]. Cells with an integer cell membrane affect the relative permittivity between two electrodes and therefore this sig-

nal is used for estimation of VCC [14, 15, 16, 17]. The model organism for application of AC measurements in the β-dispersion range is yeast, being a very important expression host for recombinant proteins [18, 19, 20]. Also approaches towards more complex expression systems, like filamentous fungi and CHO cells, are already performed [21, 22, 23, 24]. In general, these measurements show a strong dependence upon physical process parameters (like aeration and stirring - causing gas bubbles, temperature shifts and pH gradients) and are furthermore highly affected by changes in the media composition during cultivation (e.g. sugar concentrations).
However, not only high frequency impedance spectroscopy in the β-range can be used for determination of biomass, but also changes of the double layer of cells with the electrode surface (at low frequencies in the mHz range, α-dispersion) provide valuable information. Beside the cell type itself (cell wall/membrane compositions, size and shape), many physical parameter especially in the media (pH, ion concentrations) can influence the potential distribution in the double layer [25, 26]. Furthermore, the given method is capable of detecting even very small numbers of bacteria in soil, food and feces polluted water using interdigited microelectrode designs [27, 28, 29, 30, 31, 32].These studies were only performed in very small scale with a low cell concentration. In general, a threshold in the measurement is present at low cell concentration. Exceeding this limitation, very stable signals over time were achieved in these measurements. Beside direct measurements in the broth, a modified electrode system in an interdigited design can be used [33, 34, 35]. First approaches towards process monitoring were shown by Kim et al. [36], who worked with an inline sensor used in lower frequency range between 40 Hz and 10 KHz for real time monitoring of biomass. Kim et al. showed the feasibility for measuring changes in the double layer capacitance, but no analysis of the double layer capacitance (C_{DL}) itself was performed, only discrete extracted values for distinct frequency values. Furthermore, this was done only in a batch cultivation approach and no analysis of cell physiology, which is essential for differentiation between different cell states, e.g. living/dead counting, were shown. This can lead to overestimation of the measured cell density and results in additional uncertainties during the measurement.

In this Thesis, two topics are addressed. In order to measure cell leakiness, a method for determination of the viable cell concentration had to be established. On a first approach flow cytometry (FCM) in combination with different fluorescence dyes was used for cell physiology evaluation to account for changes in the viability during cultivation. In course of this evaluation a new online probe using Electrochemical Impedance Spectroscopy was developed, which provides the possibility to measure the VCC in online mode during a cultivation. With

this background it was possible to perform a systematic and comparative study of how to permeabilize the outer membrane (OM) during *E. coli* cultivation processes. Different screening experiments in shake flasks were performed prior to using the controlled environment of a bioreactor. We monitored leakiness and also analyzed lysis to be able to distinguish between these two states. To monitor cell integrity, alkaline phosphatase (AP), a marker protein for periplasmic leakiness [37, 38], was measured and cell lysis was monitored using β-galactosidase assays [39] and flow cytometry [40] in these experiments. Finally, we combined the addition of a permeabilizing agent with heat treatment, which is known to lead to leakiness of the OM in Gram-negative bacteria [41, 42, 43, 44], resulting in a release of nearly 30 % of the periplasmic content of the cells while keeping cell lysis at a minimum.

2

THEORETICAL BACKGROUND

2.1 RECOMBINANT PROTEIN PRODUCTION

2.1.1 *Fermentation techniques and challenges in E. coli*

The Gram negative bacterium *E. coli* is currently the production host of choice for high volumetric productivity with cell densities up to 190 g/l in dry cell weight [45]. Easy and straightforward cloning strategies and known genetic and physiological properties made it an attractive host for high value - low volume products. An overview of commonly produced high value products [46] and their corresponding titers are given in Fig. 1 .

Final cell mass [g(DCW) l^{-1}]	Product	Product concentration	Ref.
OD_{525} = 120	Human growth hormone	1.08 g l^{-1}	26
20	Human insuling-like growth factor-1	600 mg l^{-1}	69
OD_{525} = 11	Human growth hormone	1.75 g l^{-1}	26
68	Human α consensus interferon	5.6 g l^{-1}	70
92	Trypsin	56 mg l^{-1}	53
55	Human interleukin 1β	2.15 g l^{-1}	50
26	Human leukocyte interferon	1×10^9 IU l^{-1}	34
77	ProteinA–β-galactosidase fusion	19.2 g l^{-1}	47
60	Human interferon α1	5.5×10^8 IU g(DCW)$^{-1}$	33
50	Mini-antibody	1.04 g l^{-1}	67
58	Human interferon α1	1.26×10^9 IU l^{-1}	12
95.5	Aprotinin–β-galactosidase fusion	2.85×10^6 U l^{-1}	52
40	Human parathyroid hormone	338 mg l^{-1}	52
OD_{600} = 100	Bovine somatotropin	2.9 g l^{-1}	35
63	β-isopropylmalate dehydrogenase	16.00 U g(protein)$^{-1}$	59
101.4	PHBg	81.2 g l^{-1}	58
175.4	PHB	65.5 g l^{-1}	17
124.6	PHB	34.3 g l^{-1}	57
OD_{660} = 134.4	Human Proapo A-I	6.0 g l^{-1}	55
59.5	Human interleukin 2	1.2 g l^{-1}	56
125	*Bacillus thuringiensis* toxin	6.6 g l^{-1}	28
36	Phenylalanine	46 g l^{-1}	8
OD_{680} = 90	Human interleukin 2	–	29
OD_{680} = 75	Human interleukin 2	3.3 g l^{-1}	41
102	*E. coli* tryptophan synthase	1.6×10^5 U g(protein)$^{-1}$	46
21	Human epidermal growth factor	60 mg l^{-1}	68
84	β-galactosidase	4600 U OD_{600}^{-1}	65
145	Penicillin acylase	6.0 U mg^{-1}	38

Figure 1: High value products produced in *E. coli* and corresponding titers according to Ref. [47, 45].

Recent progress in metabolic engineering made it possible and economically feasible to use *E. coli* for low value - high volume products like poly-hydroxy-butyrate, succinic acid, octanoic acid, aromatic compounds, ethanol and acetone as a few examples [47]. Cultivation of the bacteria to such high cell densities is challenging since some major problems had to be solved [45]. A defined growth media exhibiting the maximum non metabolic inhibiting concentrations allow

a cell density of about 15 g/l, usual for batch based approaches. Beside the inhibitory effects, concentrated growth media can cause precipitations during sterilization and fermentation. These precipitations can change the nutrient supply and can result in complications upon downstream processing and recovery.

2.1.2 *Strain BL21(DE3)*

The strain BL21(DE3) emerged from a minimum of 11 single colonization steps from the B strain of Delbrück and Luria in 1942. Phylogenetic information on this strain is given in Ref. [48] tracing back to the bacterial collection of the Institute Pasteur d'Herelle 1918. The strain is nowadays employed in production of recombinant proteins under the the control of a promoter for T7 RNA polymerase in appropriate plasmid vectors [49]. Plasmids exhibiting a T7 promotor will be transcribed upon induction with the T7 polymerase. The system is able to translate almost any DNA linked to the promotor region. The pET system given in Fig. 2 is extensively applied with *BL21(DE3)*. The T7 gene is under control of the lacUV5-promotor and transcribed upon induction with IPTG or lactose. As a certain leakiness is preset even in the uninduced state, more stringent hosts including the pLysS or pLysE are generally available. The expressed T7 polymerase leads to transcription of the target gene with protein expression level up to 50 % of the total cell protein [49].

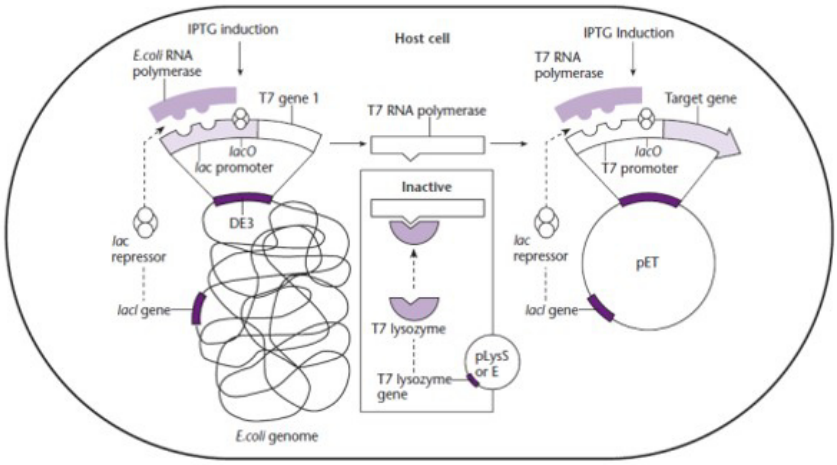

Figure 2: The pET expression system in BL21(DE3) including different genetic regulation screws for protein production (picture: https://hemankumar.wordpress.com).

Cultivations with this strain - including the pET expression system - induced with either lactose or IPTG are used in this Thesis for determination of the viable cell concentration and for monitoring the overall periplasmic leakiness.

2.2 ESTIMATION FOR VIABLE BIOMASS

For determination of the overall cell leakiness, knowledge about the degree of cell death and lysis is of outmost importance. Measurement of total biomass during bacterial cultivation is state of the art. Beside gravimetric measurements of the total biomass, like dry cell weight (DCW) and wet cell weight (WCW) and photometric approaches like OD measurement at 600 and 880 nm are used generally. In industrial processes, produced biomass estimation and closed loop control can be established through soft sensor applications [50]. However, these control systems are dependent on atline detection systems like high performance liquid chromatography for metabolite measurements. Direct cell counting is often performed using light microscope and flow chambers, indirect cell counting generally requires a prior cultivation step and is given afterwards in colony forming units (CFU/ml). However, those measurements are generally performed offline and reflect the viability of the culture only to a certain extend. In the last years new systems were established using fluorescence based methods like flow cytometry (FCM) and methods based on electrical impedance measurements of the β-dispersion effect.

2.2.1 *Flow cytometry*

Generally cultures in an ideal stirred tank reactor are regarded a uniform ensemble exhibiting the same attributes in the whole culture. This actual black box model is a rather crude approximation for a biological system since, on one hand we have basic mutational variability of cells and physiological variability as a result of the process (dropwise nutrient, base addition). A quite general statistical argument serves strikingly to highlight the heterogeneity necessarily observable in (micro)biological populations. If we assume a population that is normally distributed, only 95.45 % or 0.9545 of the population will, by definition, possess a quantitative attribute that is within +/- 2 standard deviations of the mean. This decreases below 50 % for n=5 or 15; i.e., if we consider only 15 attributes, the chance of an individual possessing normal values for each of them is less than 50 %, and the chances drop below 10 and 1 % for n= 50 and n = 99, respectively [51]. Generally, these inhomogeneities are already present in ideal stirred tank reactors. Expanding the reactor size to several m^3 causes even stronger inhomogeneity in pH, dO_2 and nutrient supply since the power input to mix the culture ideally cannot be achieved[52]. Inhomogeneity in cultures makes control strategies and model based control hard to be established. Flow cytometry is an approach to overcome these problems in measuring thousands of individual cells per second and their inherent properties and gives a fuller and more accurate view of the whole culture. A usual design is given in Fig. 3.

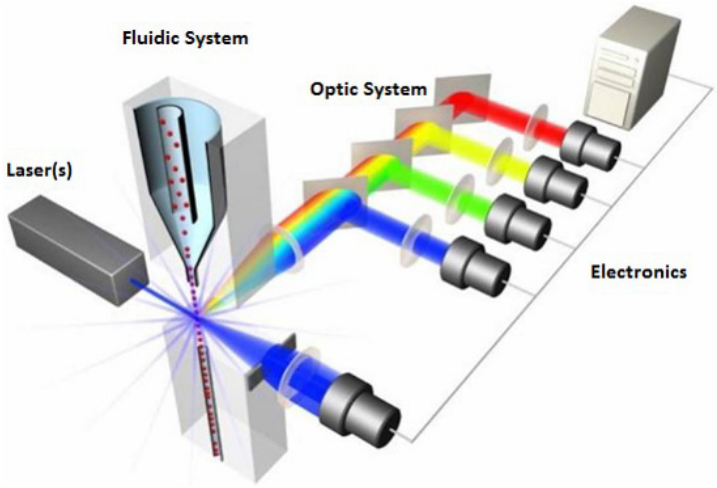

Figure 3: Principle of a flow cytometer measurement cell. Lasers with different wavelength are focused on the flowcell and excite the corresponding dye in the cells which can be measured subsequently using photo-diodes (picture: http://dx.doi.org/10.13070/mm.en.2.125)

A flow cell is used with a shear fluid to pass single cells through the analysis part. Beside forward and side scatter detection, being an indicator for shape and size of the cells, lasers with different wavelength can be applied to excite different fluorescent dyes in the sample. The signal is generally monitored using photo-diodes, which turn the photon signal into a voltage based one. These may be stains which bind to (or react with) particular molecules such as DNApH_{in} or which are taken up in response to membrane permeabilization, or, increasingly, antibodies or oligonucleotides tagged with a fluorescent probe [51]. Beside qualitative analysis of populations, cell sorting can be applied - fluorescence-activated cell sorting (FACS) - separating cells into different fractions as a function of a certain property.
Viability of prokaryontic cells at a single cell level is essential to monitor the influence of detergents and environmental conditions. A number of criteria for viability have been suggested: impermeability of the membrane to nucleic acid dyes such as propidium is one, and the presence of metabolic activity, as indicated by the production and retention of fluorescent product from a non-fluorescent enzyme substrate or by maintenance of a membrane potential, is another [53]. However, FCM can be, in the optimal case, only applied in online mode and fluorescence dyes are in general quite expensive. Therefore, approaches for direct measurement of VCC are in the focus of today's research.

2.2.2 *Electrochemical Impedance Spectroscopy (EIS)*

Measurements using the change of electrical signals as a function of the cell concentration are one promising method to develop new efficient ways for monitoring VCC on- and inline. Generally, impedance spectroscopy is an electrochemical technique with broad applications that is even further growing in importance [54]. The impedance Z is the frequency dependent alternating current (AC) resistance of a material. The impedance response can be described by a real and imaginary component:

$$Z = Z_r + jZ_j \tag{1}$$

When input and output are in phase the imaginary part of the impedance vanishes, and the impedance has only a real contribution (ohmic resistance). When input and output are out of phase by 90 %, the real part of the impedance is zero, and the impedance has only an imaginary contribution, Z_j (capacitance or inductance) as seen in figure 4.

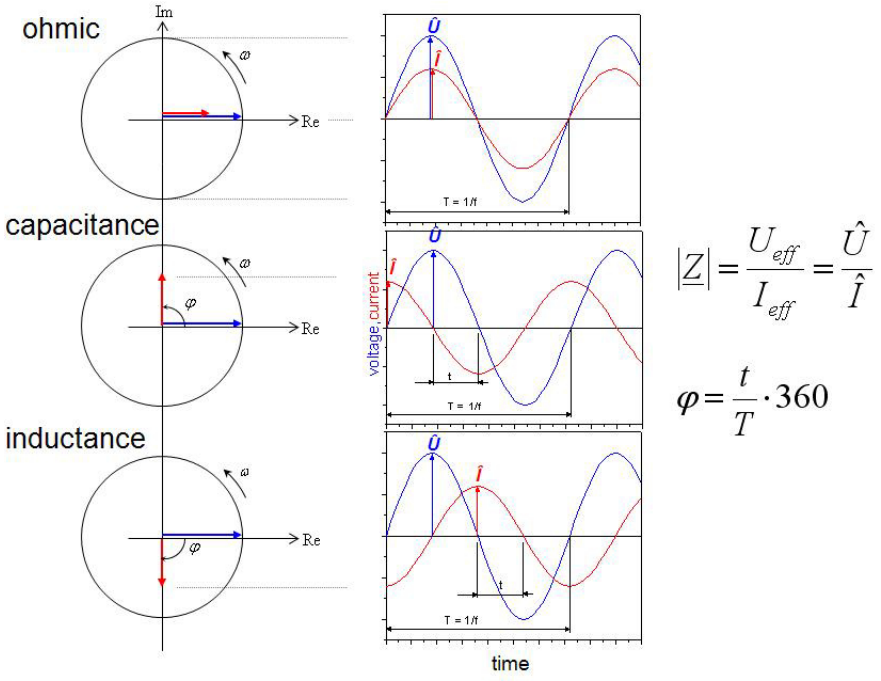

Figure 4: Principle of EIS, with three possible combinations of input and output, only ohmic resistance, capacitance, or inductance.

Impedance spectra can be visualized in multiple ways. The most important plots are the *Bode plot* and the *Nyquist plot* (see figure 5). The Bode plot uses the absolute value of the impedance (or the real and imaginary part of the impedance) versus frequency, the Nyquist plot

depicts the real part of the impedance versus the imaginary part of the impedance.

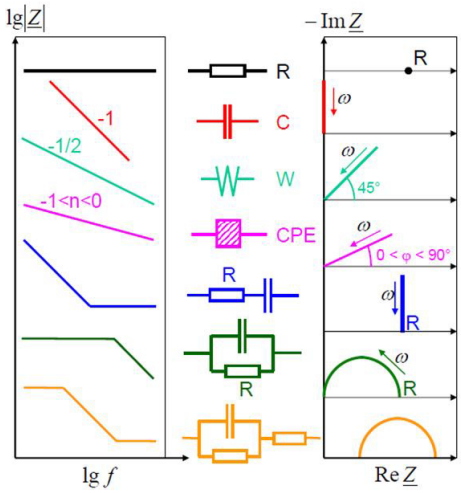

Figure 5: Bode- and Nyquist plot of for different equivalent circuit elements.

Different electrical circuits possessing the same number of time constants can yield a mathematically equivalent frequency response. Therefore constructed equivalent circuits always seek a physical meaning of the elements, and this is the major task in the analysis of impedance responses. In biological systems permittivity is in general the magnitude of choice for visualization, since dielectric properties of tissues are strongly frequency dependent [55], compare to Fig. 6 a). In general Cole-Cole plots are used according to Eq. 2

$$\bar{Z} = R_\infty + \frac{R_0 - R_\infty}{1 + (i * \omega * \tau)^\alpha} \tag{2}$$

with τ being the time constant, ω the circular frquency, R the respective resistances, and α the phase angle given in Fig. 6 b). For industrial applied measurement system the range of the β-dispersion is used in a frequency range from 20 to 0.1 MHz. The principle of the β-dispersion is the change of relative permittivity as a function of cell density. As living cells behave like small capacitances upon field load the relative permittivity is altered compared to the media alone. The reason is, that ions both inside and outside the cells can only move so far before they encounter the plasma membranes which acts as an insulating physical barrier preventing further movement, which results in the development of a charge separation or polarisation at the poles of the cells [56].
β-dispersion effects are widely exploited in systems using yeasts as model organisms, but are also spreading among cultivations using filamentous fungi and mammalian cell cultures [21, 22, 23, 24]. However, fermentation parameters, generally, affect those measurements,

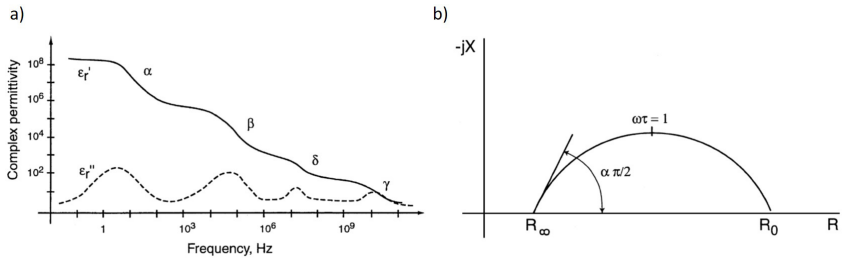

Figure 6: a) idealised dispersion regions in tissues; b) exemplary Cole-Cole plot by Ref. [55]

as the conductivity of the media, f.e., which is always an important factor of the measurement as the critical frequency of the β-dispersion is depending upon [56, 57]:

$$f_c = \frac{1}{2\pi r C_M \left(\frac{1}{\sigma_i} + \frac{1}{2\sigma_0}\right)} \tag{3}$$

with C being the capacity, σ_i the conductivity of the cytoplasm and σ_0 the conductivity of the surrounding media. An idealized measurement of a yeast cultivation of the work of Soley et. al [16] for different concentrations in the Bode plot is given in Fig. 7.

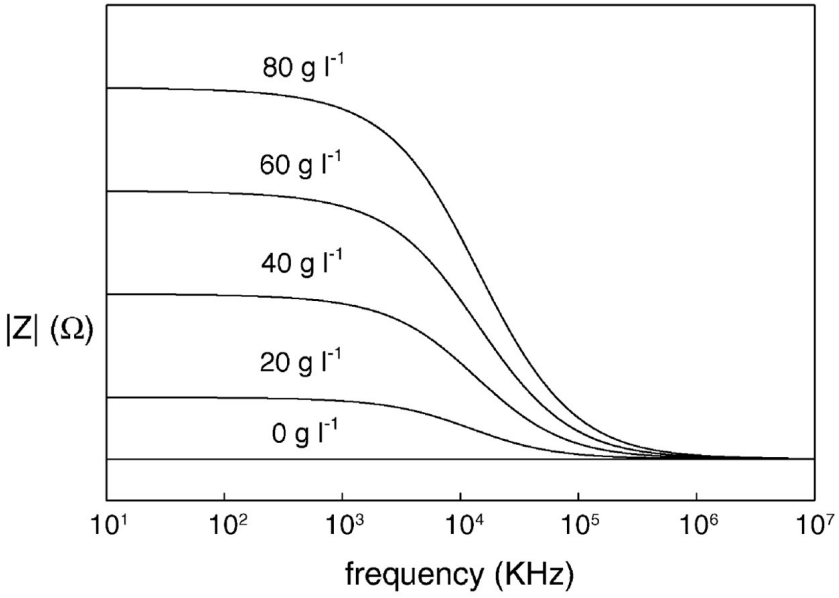

Figure 7: Signal response of different yeast concentrations between 10 kHz and 10 mHZ given in Bode plot. High frequencies reflect the media, the critical frequency is located at the turning point of the curve [16].

In general those high frequency measurements show reasonable results in systems without pronounced perturbation, like high aeration,

high stirrer speed and changes in the cultivation media as given in Fig. 7. While these perturbations are rather small in mammalian cultures, with low stirrer rates and low aeration rates, it is the limiting factor observed in microbial cultivations. Other frequency regimes, like the α-dispersion, depend mainly on electrode related effects and could be also beneficial for determination of viable biomass. A first approach towards this system is given in detail in chapter 3. With the possibility to measure online and atline viable cell concentrations, in-situ product release methods can be adequately monitored.

2.3 PRODUCT RELEASE STRATEGIES FOR GRAM NEGATIVE BACTERIA

2.3.1 *Common cell disruption methods*

The goals during cell disruption (and subsequent capture steps) of the target protein are in general a high recovery, reduced contamination and removement of cell debris. However, a certain tradeoff in those parameters is inevitable and often results in increased downstreaming costs [7]. The state of the art disruption techniques can be separated into mechanical and non-mechanical techniques (chemical lysis, freeze/thaw cycles, osmotic shock and enzymatic treatment). In industrial scale mechanical disruption - high pressure homogenizer and bead mills - are predominantly used. Fig. 2.3.1 summarizes common techniques in industrial approaches towards product recovery vs. purity for the most commonly used homogenization methods.
Cultivation (upstream processes) seems to play an important role for later cell disruption. Cells growing at high growth rate have less resistance to disruption compared to cells in slow growing or stationary phases. An increase in the production of peptidoglycans is supposed to strengthen the cell walls resulting in increased resistance to disruption [7].

2.3.2 *Secretion of target proteins into the periplasmic space of E. coli*

New product release strategies aim for changing the target location of the recombinant protein away from the cytoplasm and try to segregate the product into the periplasmic space or even into the fermentation broth. Location in the periplasmic space has several advantages. The first one is the oxidative environment, which enables correct disulfide bonding to be established in the target protein. Second, the periplasm only contains 4-8 % of the host cell proteins and only 7 out of 25 known proteases are present [59]. In general, release of periplasmic proteins is established by osmotic shock and subsequent lysozyme/EDTA treatment to completely remove the outer membrane after cultivation.

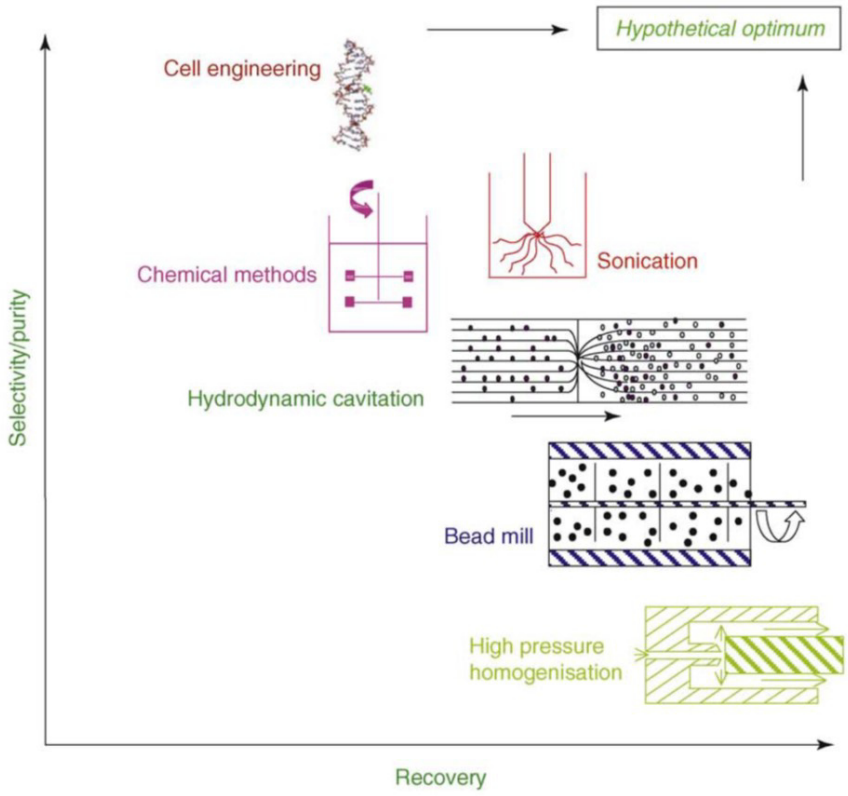

Figure 8: Overview of common mechanical and non-mechanical cell disruption techniques. Homogenization methods as a function of product recovery and purity. Products of large scale fermentation are generally processed via bead mill or high pressure homogenization according to Ref. [7, 58]

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Although formation of inclusion bodies during production of the recombinant product has certain benefits as they are highly resistant to proteolysis and can be formed in large quantities, consecutive downstream processes demand high efforts in removing contaminants and in refolding the protein. The transport to the perisplamic space or even to the fermentation supernatant can be established in several ways. *E. coli* has several transport mechanisms. The most important are shown in Fig. 9 [60]:

- Type I: an ATP-binding cassette recognizes a C-terminal signal sequences α - haemolysin (HlyA) and transports it from the cytoplasm directly to the media.
- Type II: two step transport - periplasmic and subsequent extracellular. The transport from the cytoplasm to the periplasm is mediated by the Sec-dependent or the twin-argenine-translocation (tat) pathway. In the Sec-pathway cytosolic chaperone SecB or the ribonucleoprotein signal recognition particle (SRP) bind to

the N-terminal signal peptide, in the tat-dependent a folded preprotein by TatBC complex.

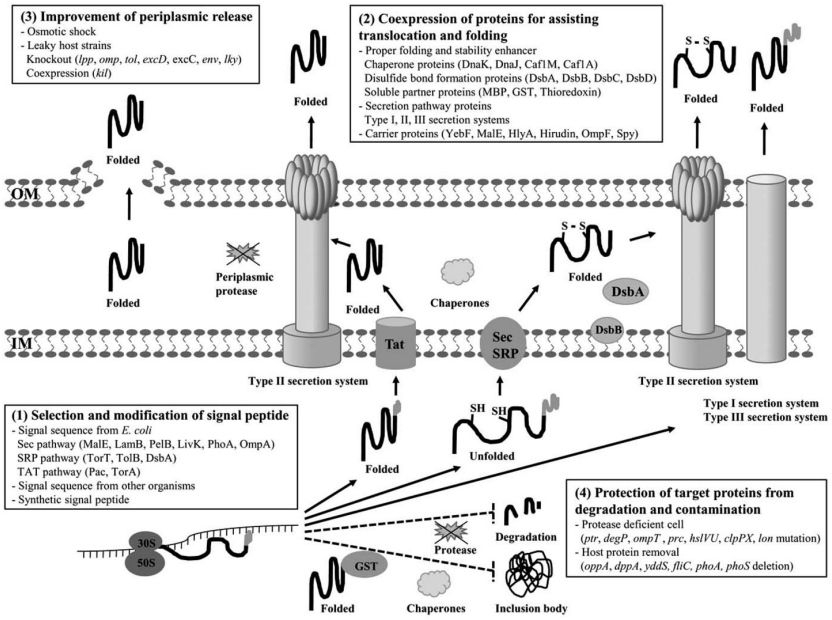

Figure 9: Secretory pathways in *E. coli* according to Ref. [60].

Focus has to be laid on the selection of the signal peptide synthesized with the target protein (fusion protein). A large number of signal peptides of different organism have already been identified using BLAST. Common signal peptides used from *E. coli* are given in Fig. 9, and are referred to the **Sec pathway** (maltose-binding protein subunit, MalE; maltoporin, LamB; pectate lyase subunit, PelB; leucine binding protein subunit LivK; alkaline phosphatase subunit, PhoA; outer membrane protein, OmpA), to the **the SRP pathway** (periplasmic sensory protein, TorT; subunit of the Tol-Pal cell envelope complex, TolB; disulfide oxidoreductase, DsbA), and to the **Tat pathway** (penicillin acylase, Pac; subunit of trimethylamine N-oxide reductase I, TorA) according to Ref. [60]. Details on the general appearance and on the amino acid sequence of the most important signal sequences can be found in Ref. [61]. Furthermore, beside the host cell signal peptides, signal peptides from other procaryonts, like *Klebsilla* and *Bacillus* and phage peptides M13, have already been used.
So one way for increasing the yield of soluble protein in the periplasm or in the fermentation medium is extension of the amino-acid sequence using signal peptides. A further approach towards increase of the yield is the co-expression of helper proteins for translocation of the cytoplasmic membrane and for proper folding of the target protein. In general two types of proteins are used: molecular chaperones and enzymes involved in disulfide bond formation [60] (compare with Fig. 9). To de-bottleneck the translocation to the periplasm,

in special the Sec-pathway, SecY and SecE are overexpressed in a wide range of recombinant *E. coli* strains.
Once translocated to the periplasm, product can be recovered by destabilization of the outer membrane. *E. coli* strains having, in general, mutations in outer membrane lipoproteine gene (lpp) [60]. The most important ones for *E. coli* outer membrane leakiness are *omp, tol, excD, excC, lpp, env, and lky*. Details on mutations and destabilization of the cell itself (*kil-gen* causing cell lysis) for different prokaryontic organism are given in detail in Refs. [62, 63]. However, those mutations severely effect the growth behavior of the cells, as the diffusion of certain components of the growth media is suppressed.

2.4 OUTER MEMBRANE COMPOSITION OF GRAM NEGATIVE BACTERIA

Knowledge of the composition of the outer membrane is essential for selective release in-situ as the given porin structures can be influenced by certain chemical detergents. Generally, gram negative bacteria, like *E. coli* are surrounded by two concentric lipid bilayer membranes, separated by and defining the periplasmic space. While the cytosolic membrane is mainly composed of phospholipids (70 - 80 % phosphatidylethanolamine, phosphatidylglycerol and cardiolipin) and is highly symmetric in both leaflet, the outer membrane (OM) is highly asymmetric. The inner leaflet has the same composition like the cytosolic membrane, while the outer membrane is composed of lipopolysaccharides with a high amount of pore-forming proteins (porins) being responsible for the overall leakiness of the OM [64, 65]. 50 % of the OM biomass consist of protein - integral bound into the membrane or attached on the N-terminus to lipids, compare to Fig. 10. Two Structural features dominate the membrane associated proteins - α -helical bundles in the cytosolic membrane and β - barrels in the OM. Out of the different topologies for β - barrels found in proteins, all OM proteins exhibit the simplest and most frequently observed one - all-next-neighbour connection between adjacent strands [64].
OmpA, OmpX and OMPLA (Phospholipase A) play mayor roles in stability and probable host interaction of the OM and do not participate in nutrient exchange. In contrast, the general porins OmpF, OmpC and PhoE are responsible for diffusion of hydrophilic molecules (<600 Da) and show no selectivity [65]. OmpA is responsible for cationic transport, while the phosphate-limitation induced PhoE acts as anionic transporter. It is supposed that charge and electrical field alterations in the porins (especially as a result of loop L3 being situated within the channel) or as result of a new transmembrane potential (removement of the hydration shell of the ions) could be responsible for the anionic and cationic selectivity and the closing of the general porins [64].

a)

Protein family	Small β-barrel membrane anchors	Small β-barrel membrane anchors	Membrane-integral enzymes	General (non-specific) porins	Substrate-specific porins	TonB-dependent receptors
Prototype protein	OmpA	OmpX	PldA (OMPLA)	OmpF	LamB	FhuA
Function	Physical linkage between OM and peptidoglycan	Neutralizing host defence mechanisms	Hydrolysis of phospholipids	Diffusion pore for ions and other small molecules	Maltose and maltodextrin uptake	Uptake of iron-siderophore complexes; signal transduction
Bacteriophages	K3, M1, Ox2, TuII*			K20	λ	T1, T5, ϕ80, UC-1
Bacteriocins	Colicin K, colicin L			Colicin N		Colicin M, microcin 25
Oligomeric state	Monomer	Monomer	Monomer/dimer	Homotrimer	Homotrimer	Monomer
Domain structure	Two co-linear domains	One domain	One domain	One domain	One domain	Two interconnected domains
Size of the membrane domain	171 residues	148 residues	269 residues	340 residues	421 residues	714 residues
PDB code[a]	1BXW	1QJ8	1QD5	2OMF	1MAL	1BY3, 2FCP
Resolution	2.5 Å	1.9 Å	2.4 Å	2.4 Å	2.6 Å	2.5 Å
Number of transmembrane β-strands, *n*	8	8	12	16	18	22
Shear number, *S*	10	8	16	20	22	24

a. PDB code, Brookhaven Protein Data Bank accession code.

b)

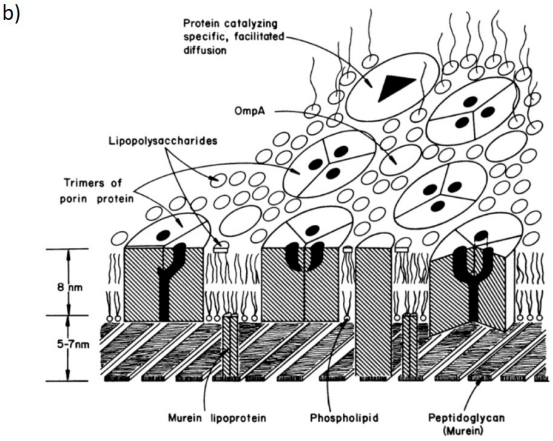

Figure 10: a) Structural and physiological properties of membrane associated proteins according to Ref. [64]. b) sketch of the OM structure of *E. Coli*, Ref. [65].

General porins show a high dependence on the external sugar concentrations. At glucose concentrations in the millimolar range, non selective porins are responsible for the transport through the OM. The relative contribution of OmpF and OmpC will be defined by the medium osmolarity, pH and growth temperature, which are only some of the factors influencing the balance of OmpF/OmpC expression [66]. Upon glucose limitation in the culture media OmpF and OmpC will drop linearly with decreasing sugar concentration. In this case, LamB which has a maltose receptor, but also affinity for glucose, will dominate the transport through the OM [66].

As already mentioned, LamB is a sugar-specific porin for maltose and maltodextrines, but exhibiting also nonspecific elements. It allows passage of amino acids, mono-saccharides and alkali ions, and increases specificity for di-saccharides and tri-saccharides. Maltose binding protein (MPB) - also used for increase of solubility in recombinant proteins - seem to increase the transport through lamB. In general, these changes in OM porin composition are regulated through the gene expression of f.e. maltose regulon. cAMP and Crp levels play a key role in these induction/repression systems.

2.5 OUTER MEMBRANE ALTERATION

One of the best studied mutational strains are deep-rough (heptose deficient) strains of *E. coli* and *S. typhimurium* - Lipopolysacceride alterations (LPS) -, which demonstrate enhanced sensitivity to a variety of hydrophobic antibiotics and detergents. In addition, they demonstrate enhanced uptake of the hydrophobic dye gentian violet, enhanced susceptibility to EDTA, Tris-lysozyme and phospholipases and leakage of periplasmic enzymes [62].
An alternative way to alter the OM permeability is the usage of detergents and antibiotics, which often result in an alteration of the LPS. EDTA, often used in combination with Tris at mild alkaline pH, removes about 30 to 67 % of the LPS in *E. coli* [62]. Other studies claim Tris at moderate temperatures to damage the outer membrane and release periplasmic proteins to the supernatant [67]. Beside these large cation species, ascorbate, benzalkoniumchlorid, serum/serum components and different antibiotics are known to affect the LPS. Details on those detergents are given in Ref. [62, 65]. A detailed analysis on new studies using a wide range of detergents is given in the following chapter using a BL21(DE)3 strain producing a recombinant protein located in the periplasm.

2.6 GOALS AND SCIENTIFIC QUESTIONS

Alterations of the OM can be achieved by different genetic and physiological modification [68]. First approaches were performed by genetically altering the porin expressing genes and, hence, produce porin deficient strains of *E. coli*. Mutations in alternative porin can result in a specific alteration in the permeability of the outer membrane, for example, maltose and maltodextran transport are preferentially reduced in mutants deficient in the Lam B porin protein [62]. However, resistance to hydrophobic and polycationic antibiotics and detergents stays unaltered. Beside the high cost for antibiotics in industrial scale cultivations, the reduced growth in those strains will lead to low time-space yields of the desired product at the end of the day. Hence, different strategies for in-situ product release are to be considered.
In a first step, measurements principles to measure the overall survivability of the cells during different treatments were tested. Beside FCM measurements, a new impedance based method was tested for determination of the viable cell concentration in online mode. With this knowledge, the overall goal of this Thesis was to evaluate the possibility of product release from the periplasmic space during a fermentation process without harvesting and centrifugation steps. Since the proposed approach should be cost saving and easily applicable, two approaches using cheap membrane permeabilizing agents and heat shocks were tested. The aim of this work can be broken down to

following scientific questions:

1st question: Can the VCC be measured reliably online during the process using FCM and an impedance spectroscopic based online probe in the α-dispersion range?

2nd question: Can the knowledge found in question 1 be used to find a detergent for selectively release of a model periplasmic protein - like alkaline phosphatase - without killing the host cell during the release process?

3rd question: Is it possible to apply the best detergent in the optimized concentration range during a fermentation process? Can synergistic effects be achieved using heat ramps and shocks during the release experiment?

Goal: Establishment of a novel principle for in situ release of periplasmic product from industrial used *E. coli* in situ. A special focus has to be brought to the viability of the cells during this process, since released proteases effect the released proteins and drastically reduce the yield of product.

Structure of this Thesis: This Thesis consists of two peer reviewed papers. Question 1 is addressed in the first paper with the title *A Novel Application for Low Frequency Electrochemical Impedance Spectroscopy as an Online Process Monitoring Tool for Viable Cell Concentrations*. Question 2 and 3 are adressed in the second one with title *How to trigger periplasmic release in recombinant Echerichia coli: A comparative analysis*.

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3

VIABLE CELL CONCENTRATION MEASUREMENTS USING EIS

This chapter is based upon the paper *A novel application for low frequency electrochemical impedance spectroscopy as online process monitoring tool for viable cell concentrations* published in Sensors in November 2016.

sensors

MDPI

Article

A Novel Application for Low Frequency Electrochemical Impedance Spectroscopy as an Online Process Monitoring Tool for Viable Cell Concentrations

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Abstract: New approaches in process monitoring during industrial fermentations are not only limited to classical pH, dO_2 and offgas analysis, but use different in situ and online sensors based on different physical principles to determine biomass, product quality, lysis and far more. One of the very important approaches is the in situ accessibility of viable cell concentration (VCC). This knowledge provides increased efficiency in monitoring and controlling strategies during cultivations. Electrochemical impedance spectroscopy—EIS—is used to monitor biomass in a fermentation of *E. coli* BL21(DE3), producing a recombinant protein using a fed batch-based approach. Increases in the double layer capacitance (C_{dl}), determined at frequencies below 1 kHz, are proportional to the increase of biomass in the batch and fed batch phase, monitored in offline and online modes for different cultivations. A good correlation of C_{dl} with cell density is found and in order to get an appropriate verification of this method, different state-of-the-art biomass measurements are performed and compared. Since measurements in this frequency range are largely determined by the double layer region between the electrode and media, rather minor interferences with process parameters (aeration, stirring) are to be expected. It is shown that impedance spectroscopy at low frequencies is a powerful tool for cultivation monitoring.

Keywords: *Escherichia coli*; viable cell count; online biomass monitoring; impedance spectroscopy

1. Introduction

Microbial cultivations play a key role in many different fields such as food, drug and bulk chemical production as well as in waste to value concepts [1]. Process monitoring such as pH, dO_2, offgas analysis and biomass measurements are state of the art in today's industrial cultivations to guarantee product quality and safety. Generally, in industrial processes, produced biomass estimation and closed loop control can be established through soft sensor applications [2]. However, these control systems are dependent on atline detection systems such as high performance liquid chromatography for metabolite measurements. Therefore, the accurate and reliable measurement of biomass [3,4] and especially of

viable cell concentrations (VCC) during cultivations increases the accuracy of given input parameters and increases the efficiency of these process control tools.

VCC is measured using offline measurement principles including marker proteins or fluorescence probes, such as flow cytometry or confocal microscopy [5,6]. Online and inline approaches are rather scarce and are based on physical measurement principles. One principle generally applied is high frequency alternating current (AC) impedance spectroscopy with high field amplitudes used on the basis of the so called β-dispersion [7,8]. Cells with an integer cell membrane affect the relative permittivity between two electrodes and therefore this signal is used for the estimation of VCC. A detailed description of the measurement principles can be found in References [9–12].

The model organism for the application of AC measurements in the β-dispersion range is yeast, as it is a very important expression host for recombinant proteins [13–15]. Also, approaches towards more complex expression systems, such as filamentous fungi and chinese hamster ovary (CHO) cells, are already performed [16–19]. In general, these measurements show a strong dependence upon physical process parameters (such as aeration and stirring—causing gas bubbles, temperature shifts and pH gradients) and are furthermore highly affected by changes in the media composition during cultivation (f.e. sugar concentrations).

However, not only high frequency impedance spectroscopy in the ß-range can be used for the determination of biomass, but also changes of the double layer of cells with the electrode surface (detectable at low frequencies in the mHz range, α-dispersion) provide valuable information. Beside the cell type itself (cell wall/membrane compositions, size and shape), many physical parameters, especially in the media (pH, ion concentrations), can influence the potential distribution in the double layer [20,21]. Furthermore, the given method is capable of detecting even very small numbers of bacteria in soil, food and feces-polluted water using interdigitated microelectrode designs [22–27].

These studies were only performed at a very small scale with a low cell concentration. In general, a threshold in the measurement is present at a low cell concentration. Exceeding this limitation, very stable signals over time were achieved. Beside direct measurements in the broth, a modified electrode system in an interdigitated design can be used [28–30]. First approaches towards process monitoring were shown by Kim et al. [31], who worked with an inline sensor used in the lower frequency range between 40 Hz and 10 kHz for real-time monitoring of biomass. Kim et al. showed the feasibility for measuring changes in the double layer capacitance, but no analysis of the double layer capacitance (C_{dl}) itself was performed; only discrete extracted values for distinct frequency values were used. Furthermore, this was done only in a batch cultivation approach and no online or offline analysis of cell physiology, which is essential for differentiation between different cell states, e.g., living/dead counting, were shown. This can lead to overestimation of the measured cell density and results in additional uncertainties during the measurement.

In this study, impedance measurements during a fed batch–based cultivation, as used in industrial-scale bioreactors, with *E. coli* BL21(DE3), producing a recombinant cytosolic protein, are presented. For calibration of the signal, offline and online measurements of the impedance are correlated to measured biomass. Different state-of-the-art methods are applied for determination of the corresponding total biomass—dry cell weight (DCW), OD_{880} inline and OD_{600} offline. Flow cytometry (FCM) in combination with different fluorescence dyes is used for cell physiology evaluation to account for changes in the viability during cultivation. With this knowledge, we are able to correlate the total biomass to the extracted double layer capacitance.

Within this study, a prototype online probe (flow-through cell) was designed and built. With this easy-to-rebuild probe we show an excellent correlation between double layer capacitance and viable cell concentration which allows online cell concentration monitoring with high accuracy over a very broad cell concentration (1 g/L to 40 g/L investigated in this study).

2. Materials and Methods

2.1. Expression Host and Cultivation

All cultivations were performed using an *E. coli* BL21(DE3) strain as expression host transformed to produce recombinant horseradish peroxidase (HRP) (pet39+/HRP) or a recombinant cytoplasmic antibody fragment. For the preculture 500 mL sterile DeLisa medium was inoculated from frozen stocks (1.5 mL, −80 °C) and incubated in a 2500 mL High-Yield shake flask for 20 h (230 rpm, 37 °C). Batch and fed batch cultivations were performed in a stainless steel Sartorius Biostat Cplus bioreactor (Sartorius, Göttingen, Germany) with 10 L working volume. A batch and fed-batch phase for biomass generation were followed by an induction phase using a mixed feed medium with glucose as primary carbon source and lactose as carbon source as well as inducer. Detailed information about the bioreactor setup and media composition can be found elsewhere [32].

2.2. Analytics

For DCW measurements 2 mL of the cultivation broth was centrifuged at 4500× *g*, subsequently washed with 0.9% NaCl solution and centrifuged again. After drying the cells at 105 °C for 48 h the pellet was evaluated gravimetrically. DCW measurements were performed in triplicates and the mean error for DCW was always 3%. Offline OD_{600} measurements were performed in duplicates in a UV/VIS photometer Genisys 20 (Thermo Scientific, Waltham, MA, USA).

For inline OD_{880} measurements a Dencytee total cell density measurement cell (Hamilton, Reno, NV, USA) was used. In general, at cell densities above 20 g/L (DCW) saturation effects were observed [33] (exceeding linear range of Lambert Beer's law as already observed for offline OD_{600} measurements). Verification of cell viability was done by flow cytometric (FCM) measurements. After addition of DiBAC4 (bis-(1,3-dibutylbarbituricacid)trimethineoxonol) and Rh414 dye diluted cultivation broth was measured using a CyFlow Cube 8 flow cytometer (Sysmex-Partec, Bornbach, Germany). Rh 414 binds to the plasma membrane and visualizes all cells, while DiBAC is sensitive to plasma membrane potential and therefore distinction between viable and non-viable cells can be achieved. Detailed information on the viability assay can be found elsewhere [34]. Overall errors with this method were in the range of 0.5% to 1%. As less than 5% of dead cells were detected in all samples, DCW and VCC can be assumed to be equivalent.

3. Results

Within this study we developed a method to estimate viable cell concentration by measuring low frequency electrochemical impedance spectra during cultivation. Therefore, we (1) constructed a prototype probe and developed a method to link the impedance signal to the viable cell concentration. As the signals of the physical measurement probes are often dependent on changing process parameters and media composition; (2) we investigated the impact of changing the media background on the measurement during the cultivation. Finally; (3) we implemented the constructed impedance probe in the online mode to show the feasibility of this novel biomass sensor.

3.1. Construction of the Prototype Online Probe and Data Processing

Before the construction of a prototype probe, samples were measured in the offline mode by pipetting the samples into a glass cuvette with incorporated electrodes for measuring the capacitance signal. Stainless steel electrodes were used as they are described in literature to give good signals for analyzing bacteria [27]. For online measurements, we constructed a thermostatically controlled flow cell, which was connected to the bioreactor by a peristaltic pump and automatically recorded the signal in regular time intervals. The flow cell and the experimental setup for online application are presented in Figure 1.

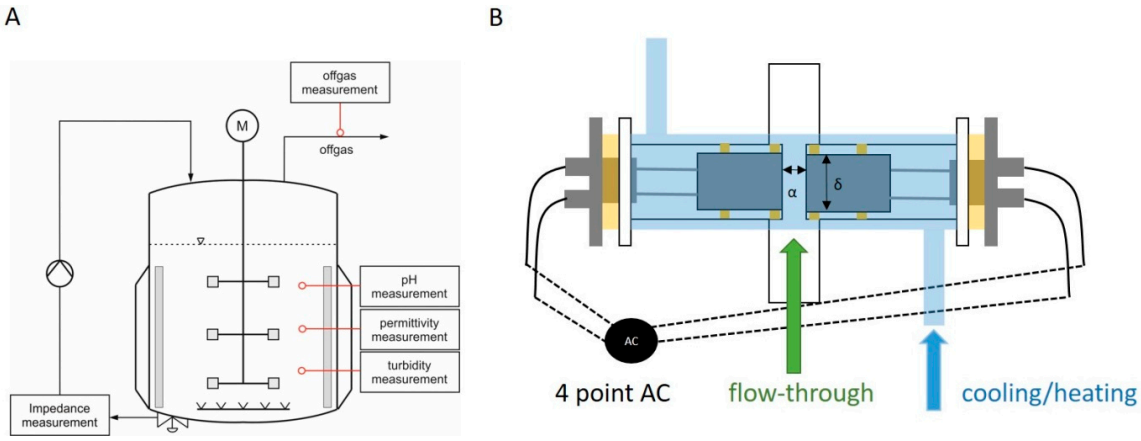


Figure 1. (**A**) Schematic drawing of the fermentation setup using the online impedance probe. A peristaltic pump establishes the flow through the probe; (**B**) Schematic drawing of the prototype online probe during fermentation. The stainless steel electrodes are sealed in borosilicate tubing (NW16KF) and a polymethymetacrylate mantle. An electrical connection is established using a four-point measuring method to decrease cable induction and setup interferences.

To facilitate rebuilding the online probe, commercially available standardized parts were used. The flow cell was made of a borosilicate glass cylinder using DN 16 glass cylinders with an adaptor connection. The continuous flow through the flow cell was maintained by connecting a peristaltic pump to the sampling port and pumping the cultivation broth continuously through the probe (exceeding 100 mL/min). The outer heating/cooling jacket was made of polymethymetacrylate cylinders and boards and allowed temperature control of the probe by connecting the double jacket to a heating and cooling thermostat alpha-RA (Lauda, Lauda-Königshofen, Germany). We chose the distance between the electrodes to be α = 1.4 cm (see Figure 1) to guarantee a stable and fast flow through the probe without pressure loss. The diameter of the circular electrodes was designed to be δ = 1.3 cm (see Figure 1) to fit into the DN16 glass cylinders. Electrical contact was established by soldering onto commercial available "Bayonet Neill Concelman" (BNC) cables.

Physical analysis of VCC in state-of-the-art capacitance probes, which rely on β-dispersion (10^7 Hz–10^4 Hz), showed high dependence on process parameters (e.g., stirring, temperature, pH, salt and substrate concentration, etc.) and the cultivation phase (exponential growth phase, starvation phase, etc.) [11,19]. We focused the measurement on a different physical phenomenon (α-dispersion), which yields valuable information mainly about biomass concentration. The so called α-dispersion effect, at frequencies below 10 kHz, which is most probably a result of the deformation of ionic species around the cell membranes, is used for these measurements. The dielectric response is therefore proportional to the ionic charge gathered around the membrane of adsorbed cells on the electrode [20,21]. Impedance measurements were recorded in the range of 10^6 to 10^{-1} Hz with amplitudes between 100 and 500 mV using an Alpha-A High Resolution Dielectric Analyzer or a Pot/Gal measuring interface (Novocontrol, Montabaur, Germany). Since measurements in this frequency range are largely determined by the double layer region between the electrode and media, rather minor interferences with process parameters (aeration, stirring) were to be expected.

To show the feasibility of monitoring the biomass concentration by impedance spectroscopy, *E. coli* cells were measured at different concentrations (Figure 2).

An obvious increase of the capacitance (arc bending to the right) in the Nyquist plot is visible between high and low biomass concentrations (Figure 2A), which proves the applicability of the chosen method to differentiate between different cell concentrations.

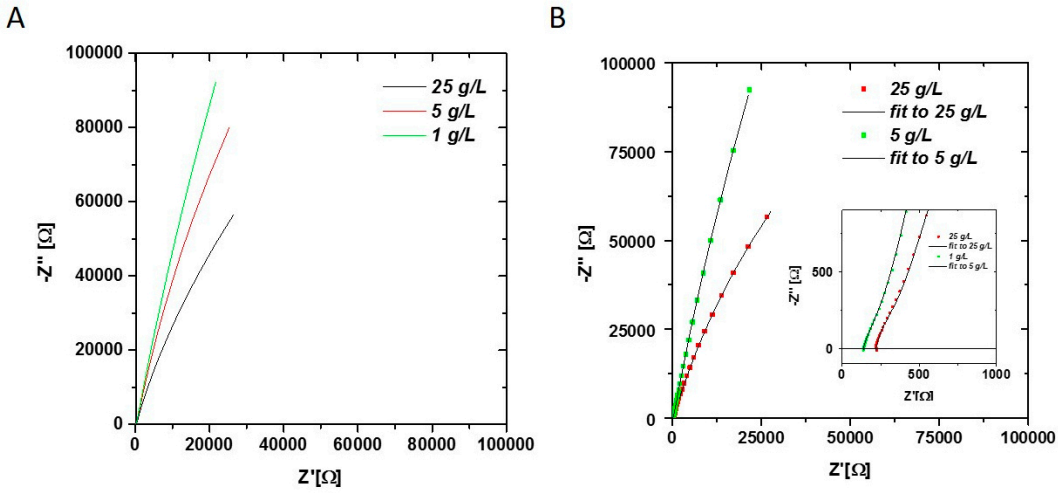
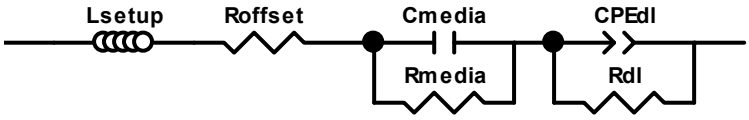


Figure 2. (**A**) Nyquist plot of *E. coli* samples at different concentrations (1–25 g/L) measured in offline mode. Obvious changes in the double layer are visible at low frequencies; (**B**) Nyquist plot of 1 g/L and 25 g/L sample with fit using equivalent circuit in Equation (1). Fitted parameters all three samples are given in Table 1. The Q value of the CPE_{dl} element is strongly dependent on the biomass concentration.

To explain the shape of the Nyquist plot and also to obtain parameters which can be used to establish a correlation between the cell concentration and impedance signal, the following equivalent circuit, including the media contributions [25], was chosen to mechanistically describe the data (Equation (1)).



(1)

The circuit contains an inductance and a resistance term for setup correction (L_{setup} and R_{offset}), a resistance-capacitance element for the media contributions (R_{media}-C_{media}) (high frequency shoulder visible in the Niquist plot—inlay Figure 2B) and a parallel connection of the resistance and constant phase element for the double layer contribution (R_{dl}-CPE_{dl}).

The impedance (Z) of a general resistance and a non-ideal capacitance R-CPE element (the connection of a resistor (R) and a constant phase element (CPE)) can be expressed by Equation (2).

$$Z = \frac{1}{(i\omega)^n Q} \tag{2}$$

where ω is the arc frequency and i is the imaginary number; n and Q are parameters that need to be fitted and are used to calculate the sample capacitance (C) according to Equation (3) [35].

$$C = \left(R^{1-n}Q\right)^{1/n} \tag{3}$$

At high cell densities, R_{dl} can be fitted and reflects changes in the ionic composition of the double layer. At low cell concentrations, R_{dl} is not accessible due to a high overall fitting error (see Table 1) and leads to a high error of the calculated sample capacitance (C) according to Equation (3).

The data obtained from the impedance measurements of the offline biomass samples were fitted according to the proposed equivalent circuit shown in Equation (1) by complex non-linear least square fitting (CNLS) using the software ZView (Scribner, Southern Pines, NC, USA). The results for the fit (displayed in Figure 2B) and the corresponding parameters are given in Table 1.

Table 1. Fitting results of CNLS fit given in Figure 2B with corresponding error estimations (Chi squared: 1.1825×10^{-3}; Sum of squares: 0.1596) and calculated real capacitance according to Equation (3).

	L_{offset} [H]	R_{offset} [Ω]	R_{media} [Ω]	C_{media} [F]	R_{dl} [Ω]	CPE_{dl}-Q [Fs^{n-1}]	CDP_{dl}-n [-]	C_{dl} [F]
abs. value for 25 g/L	2.18×10^{-6}	218.6	78.6	1.66×10^{-5}	3.95×10^{5}	2.14×10^{-5}	0.82	3.42×10^{-5}
NRMSE [%] for 25 g/L	10.70	0.19	5.10	4.24	4.85	0.42	0.19	-
abs. value for 1 g/L	1.83×10^{-6}	139.6	83.69	1.15×10^{-5}	4.44×10^{6}	1.62×10^{-5}	0.87	3.07×10^{-5}
NRMSE [%] for 1 g/L	8.48	0.20	3.31	2.67	38.23	0.38	0.15	-

The CPE-n value is a marker for the non-ideality of the corresponding capacitance. This value can be evaluated from the experimental data and Equation (1), and is mostly in an acceptable range between 0.8 to 0.9 (vs. 1.00 for an ideal capacitance) to warrant calculation of a reasonable C_{DL} value. Therefore, we used CPE_{dl}-Q values instead of the calculated sample capacitance for establishing a correlation between cell concentration and impedance data to reduce the error and extend the applicability of this measurement method also to low cell concentrations, where R_{dl} has a high error. All fitted parameters give physically reasonable values and a low NRMSE between 0% and 10% (except for R_{dl} at low biomass concentrations) which indicates that the proposed model (Equation (1)) is valid. For all further analysis, CPE_{dl}-Q was used to correlate the viable cell concentration to the impedance spectra.

3.2. Impact of Changing Media Background on Impedance Measurements

As already shown, the developed method is capable of measuring changes in the cell concentration as a function of the double layer capacitance. To apply this method also in the online mode, the impact of the media composition, which generally changes during a cultivation, on the measurement was investigated by measuring samples in the offline mode. Therefore, (1) a cultivation medium with different sugar concentrations; (2) centrifuged cultivation supernatants at different process times with different salt, sugar and host cell protein concentrations; (3) cultivation broth with cells at different process times; and (4) cells resuspended in the same matrix (cultivation medium) at different cell concentrations were analyzed.

For determination of the interferences of sugars with the measurement, cultivation media supplemented with up to 200 g/L of glucose, lactose and galactose were tested. Differences in the monosaccharide concentration seem to have no effect on the measured signal. Only at very high concentrations of lactose (200 g/L) minor changes (not shown) in the double layer capacitance were observed, but these high concentrations usually do not occur during *E. coli* cultivations.

To investigate the impact of produced metabolites, changing salt and sugar concentration samples were taken throughout a bioreactor cultivation which yielded up to 30 g/L DCW biomass.

The resulting double layer capacitances for the centrifuged cultivation supernatants (circles), for cultivation broth samples (squares), and for resuspended cells (triangles) are shown in Figure 3 as a function of DCW. FCM measurements of all samples within this study were performed to verify the viability of the cells. As less than 5% percent of the cells were dead throughout all samples, DCW and VVC are equivalent.

The composition of the cultivation supernatant changes as a function of time due to different cell concentrations. The impedance signal of the clarified fermentation supernatant is plotted as a function of the biomass concentration of the samples before centrifugation in Figure 3 (red circles). The slope of the curve is very low ($k = 6.4 \times 10^{-8}$), indicating a low impact of the medium on the overall impedance signal.

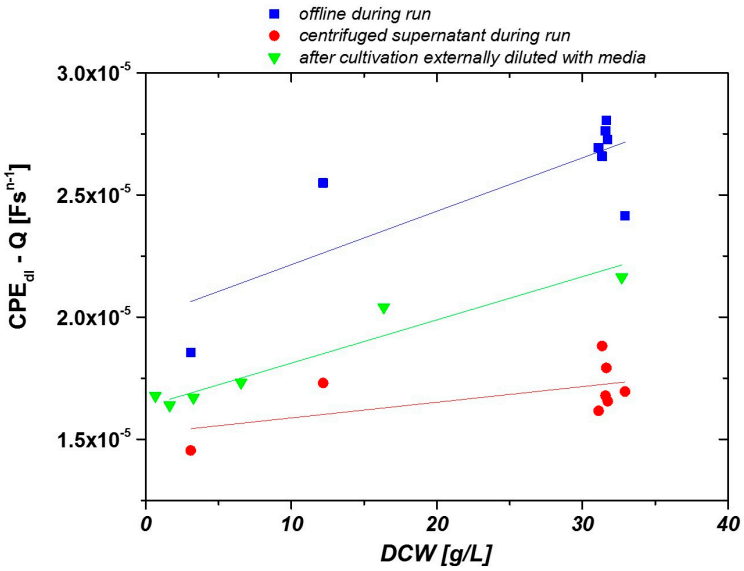


Figure 3. Offline measurements of clarified fermentation supernatant (**red circles**), cultivation broth (**blue squares**) and harvested cells resuspended in buffer at different concentrations (**green triangles**).

Resuspending centrifuged biomass in cultivation media at different concentrations resembles a significant linear fit between the biomass concentration ($k = 1.8 \times 10^{-7}$) and impedance signal (Figure 3, green triangles). While cultivation samples with cells were measured in the offline mode (blue squares), the fit of the calibration curve was not as nice as for the calibration made from diluted samples. We believe that this noise is a result of different storage times between sample taking and measuring the samples, as storing of the samples can have a severe impact on the morphology of the cells and thus on the impedance signal [36]. Furthermore, temperature control was not possible and sedimentation of the cells might have impacted the measurements. Therefore, we decided to construct a prototype with temperature control and a constant flow-through as described above and measured samples in the online mode.

3.3. Impedance Measurement in Online Mode Using the Developed Prototype

To remove noise in the impedance, the signal due to storage of the samples between sample taking and measurements, the different temperatures during analysis and the sedimentation of the cells, the developed prototype is installed in the online mode as shown in Figure 1.

Deviations of the ideal impedance signal compared to offline measurements can be found in flow-through mode. Measurements at 100 to 300 mV amplitude of the media without cells showed loops to negative differential resistances at lower frequencies (Figure 4). This indicates a slightly changing double layer resistance R_{dl} over the observed recording time of one impedance spectrum, respectively.

Since those impedance responses can already be spotted before inoculation, it might be related to a charge transfer reaction of media components on the electrode. An electrolyte-related change in the stability of the electrode may lead to passivation reactions. These can result in those negative differential resistances (changes of the current in respect to the applied voltage) [37]. Furthermore, negative resistances can occur during absorption/reactions on the electrode [38]. Similar impedance responses are obtained, for example for glucose, with Ni-containing electrodes [39]. Since the used stainless steel electrodes contain Ni as well, such reactions may also be possible in this case. However, since such negative resistances are not found during offline measurements of sugars, flow-through during the measurements seems to have a major effect on the spectra. Rather high amplitudes of 500 mV do not show the pronounced behavior seen at lower amplitudes and are therefore used for the later measurements in flow-through mode (Figure 4).

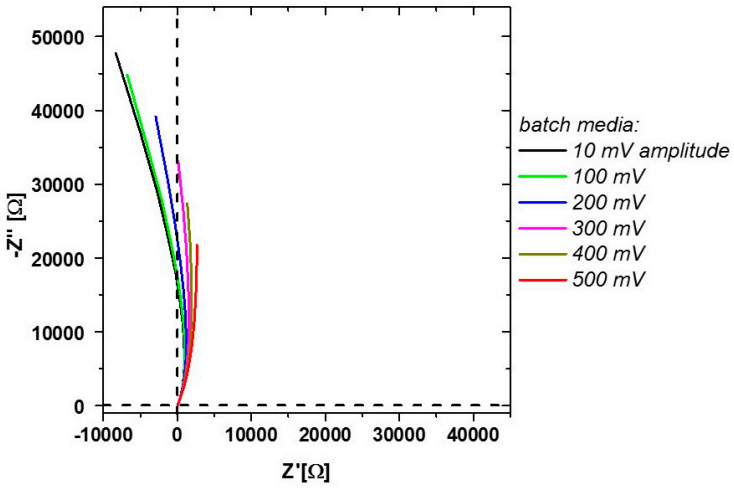


Figure 4. Measurement during flow-through including changes in the measured media sample. Higher amplitudes shift the differential resistance to positive values.

Furthermore, DCW, OD_{600} offline and OD_{880} inline were monitored. To show linearity between DCW and OD_{600} offline, and OD_{880} inline and the impedance signal, the correlation between those signals is plotted in Figure 5 using OriginLab software (OriginLab Corporation, Northampton, MA, USA). In general, media contributions are negligible with increasing electrode distance α during these measurements. Hence, values for the double layer capacitance could also be determined even with simplified fitting routines using a resistance for the real axis offset and a CPE element for the double layer capacitance when R_{dl} is far too high, especially in the beginning of the cultivations.

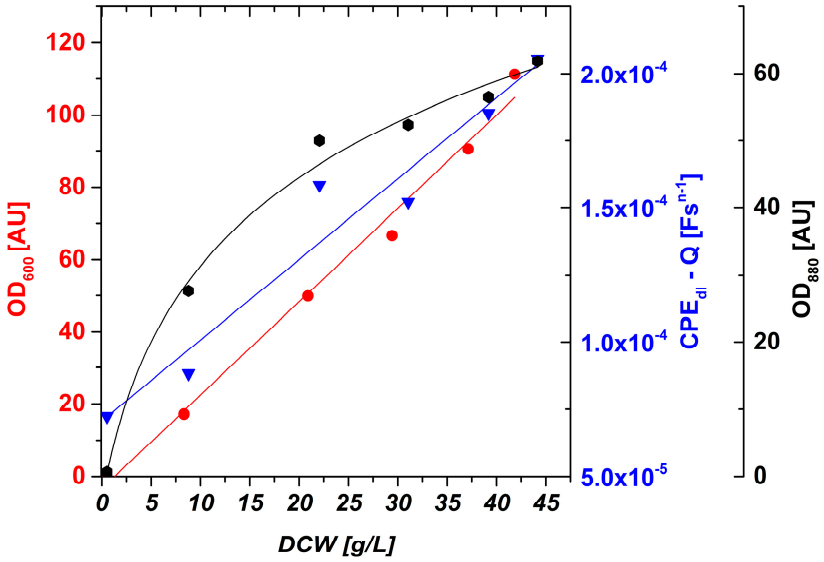


Figure 5. OD_{600} offline, OD_{880} inline and impedance online (CPE_{dl}-Q) signals as a function of DCW. OD_{600} online and CPE_{dl}-Q online show a linear behavior and were thus fitted by a linear regression. OD_{880} inline shows saturation at higher DCW and was thus fitted by a logarithmic curve.

OD_{880} inline shows a linear behavior up to about 20 g/L DCW. At higher biomass concentrations, saturation effects according to Lambert-Beer's law occur and result in a nonlinear fit with low sensitivity. OD_{600} offline shows a good linear fit also at high DCW, but samples have to be taken and processed (diluted) and the signal is not available in the online mode. The CPE_{dl}-Q online signal shows a high linearity (R^2 = 0.94) in a very dynamic biomass concentration range.

To show the reproducibility of the developed measurement principle, a correlation between DCW and CPE_{dl}-Q was established for two different cultivations (Figure 6).

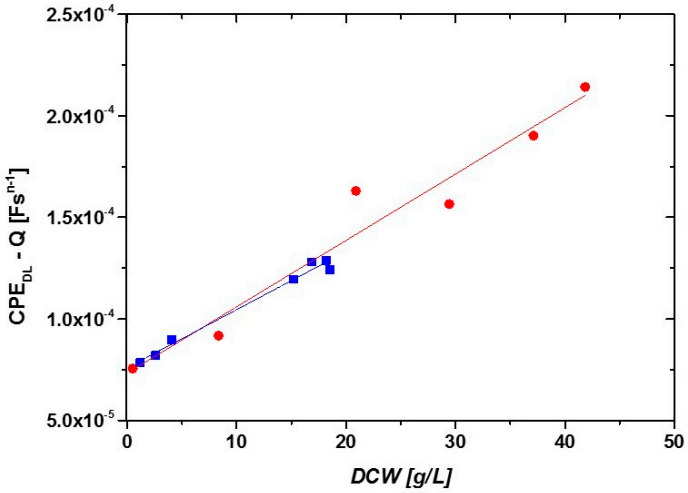


Figure 6. Correlation between DCW and impedance signal (CPE_{dl}-Q) measured in online mode during two fed batch cultivations with different feeding strategies.

Both fits show very similar slopes (Figure 6) and a high R^2 (0.94 and 0.98). The correlation between DCW and CPE_{dl}-Q was used to calculate the DCW using the impedance signal for two different cultivations exhibiting different specific growth rates, μ. Figure 7A shows the measured and the calculated DCW as a function of time for the two different cultivations and Figure 7B shows the correlation between calculated and measured DCW.

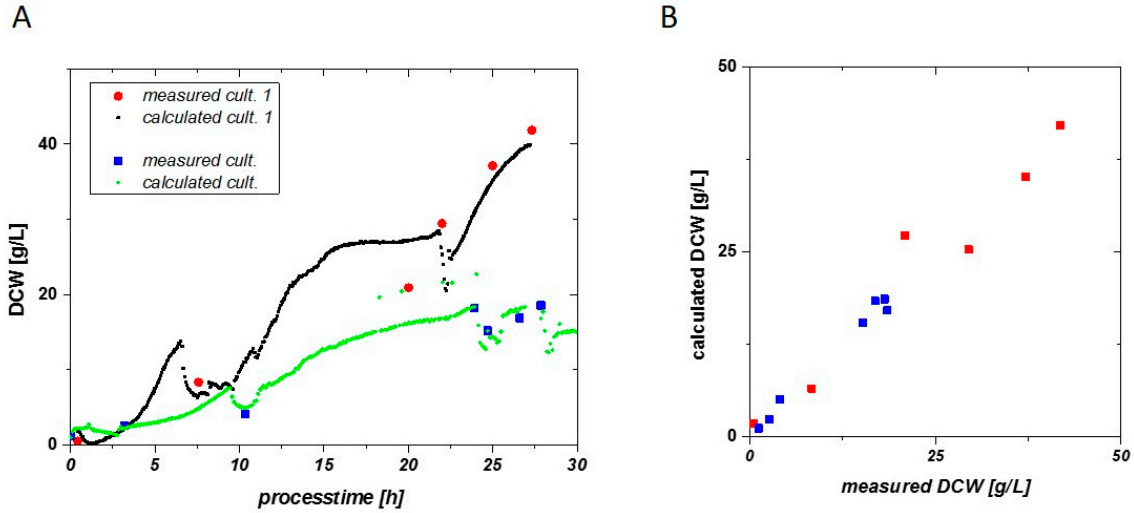


Figure 7. (**A**) DCW can be calculated by the online impedance signal very accurately. Determination of the VCC via double layer capacitance is reproducible for different *E. coli* cultivations; (**B**) Calculated DCW vs. measured DCW.

Very good reproducibility is found in two cultivations, even with very different specific growth rates applied. The quality for process control strategies can further be highlighted in Figure 7B. The calculated DCW vs. the measured DCW is situated along the first median. Values not situated along the first median indicate for the overall error in the fitting routine, compared to a residual analysis. Only measurements in the late fed batch phase of cultivation 1 are way off the first median and result in an overestimation of biomass at this time point.

4. Conclusions

Within this study we presented the feasibility of measuring DCW of *E. coli* using impedance spectroscopy at low frequencies. The proposed technique is easily applicable and has a high dynamic range from low cell densities at the beginning of the batch phase to cell densities beyond 40 g/L DCW. It is shown that this technique can be applied in an industrial fermentation strategy with high resolution and high reproducibility. Furthermore, the developed prototype can be easily rebuilt, as standardized parts were used. We believe that this measurement technique will greatly facilitate bioprocess development as VCC can be measured in real time at low but also at high cell densities with low background interferences.

Author Contributions: G. Brunauer was responsible for the prototype setup, D. Wurm; C. Slouka: O. Spadiut and C. Herwig performed the cultivation experiments, A. Welzl, C. Slouka and J. Fleig evaluated data and corresponding model.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

VCC—viable cell concentration, FCM—flow cytometry, AC—alternating current, OD—optical density, DCW—dry cell weight, C—capacitance, CPE_{dl}—constant phase element of the double layer, R_{dl}—double layer resistance, R_{offset}—offset resistance, L_{setup}—inductivity of the measurement setup, Z—impedance, ω—arc frequency, NRMSE—root-mean-square-deviation.

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4

PERIPLASMIC RELEASE IN E. COLI

This chapter is based upon the paper *Comparative analysis of how to trigger periplasmic release in recombinant E. coli bioreactor cultivations* published in ELS, August 2016.

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Research Article

How to trigger periplasmic release in recombinant *Escherichia coli*: A comparative analysis

Recombinant protein production in *Escherichia coli* usually leads to accumulation of the product inside the cells. To capture the product, cells are harvested, resuspended, and lysed. However, in cases where the product is transported to the periplasm, selective disruption of the outer membrane leads to much purer crude extracts compared to complete cell lysis, as only 4–8% of the native *E. coli* host cell proteins are located in the periplasmic space. A variety of different strategies to enable selective release of the product from the periplasm is available. However, in most of these studies cells are harvested before they are resuspended in permeabilization agent and no differentiation between leakiness and lysis is made. Here, we tested and compared different strategies to trigger leakiness. In contrast to other studies, we performed these experiments during cultivation and quantified both leakiness and lysis. In summary, we recommend incubation with 350 mM TRIS at constant pH for several hours followed by a mild heat treatment up to 38°C to trigger leakiness with only minimal lysis. This study represents a comparative summary of different strategies to trigger *E. coli* leakiness and describes a solid basis for further experiments in this field.

Keywords: *Escherichia coli* / Lysis monitoring / Outer membrane integrity / Recombinant protein production / Selective periplasmic release

Additional supporting information may be found in the online version of this article at the publisher's web-site

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1 Introduction

Escherichia coli is one of the most widely used hosts for recombinant protein production as it can be cultivated on cheap minimal media to high cell densities, straight forward cloning procedures are available and high productivities can be achieved [1–5]. As *E. coli* usually does not secrete proteins to the culture medium, recombinant products are usually located inside the cell [6]. For product recovery, cells have to be harvested, resuspended, and lysed. However, cell lysis does not only release the product but also unwanted impurities such as DNA, host cell proteins, and proteases [7].

Due to the intrinsic feature of having disulfide bonds, some proteins need to be exported to the oxidative environment of the *E. coli* periplasm. In such a case, selective disruption of the outer membrane (OM) leads to much purer crude extracts compared to complete cell lysis, as only 4–8% of the native *E. coli* host cell proteins are located in the periplasmic space [8]. Different approaches to destabilize the OM of *E. coli*, either during cultivation (DC) or post harvesting (PH), are summarized in Table 1.

As shown in Table 1, several strategies to selectively release proteins from the periplasm were tested. The treatment with guanidine HCl and Triton in varying concentrations described a successful strategy leading to a release of up to 90% of periplasmic proteins [9, 10]. However, all the studies shown in Table 1 lack information about the integrity of the cells and do not

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Abbreviations: AP, alkaline phosphatase; **β-gal**, β-galactosidase; **BaCl**, benzalkonium chloride; **DC**, during cultivation; **FCM**, flow cytometry; **HRP**, horseradish peroxidase; **OM**, outer membrane; **PH**, post harvesting; $\mathbf{q_{s,glu}}$, specific glucose uptake rate

*Both authors contributed equally to this work.

Table 1. Summary of approaches to release periplasmic proteins from *E. coli*

DC/PH	Permeabilization agent/ method	Comments	Reference
PH	Guanidine HCl and/or Triton	Resuspension of cells in TRIS after harvesting, different combinations of Triton X-100 and guanidine HCl	[9, 10]
PH	Cernitrate	Resuspension of cells in saline after harvesting	[11]
PH	Benzalkonium chloride	*Salmonella typhimurium*	[12]
PH	Glycol ethers	*E. coli* and *P. fluorescens*, two phase extraction with different glycol ethers	[13]
PH	Chloroform	*Salmonella TA831*, resuspension of cells in TRIS after harvesting	[14]
PH	TRIS	*E. coli D280* and *F515*	[15]
DC	1% Glycine	α-Amylase release from *E. coli HB101/pHI30*	[16]
PH	Polyethylenimine	*E. coli, P. aeruginosa,* and *S. typhimurium*	[17]
DC	Polyethylenimine	Addition during cultivation aiming at improving protein/DNA ratio	[18]
DC/PH	Urea/10 mM DTT	Two-phase extraction with polymer and salt at pH of 10	[19]
PH	Mild heat shock + TRIS	Resuspension of *E. coli* cells in TRIS buffer in combination with heat shock	[20–22]
PH	Osmotic shock	Harvesting, washing, equilibrating, and resuspending	[7]

DC, during cultivation; PH, post harvesting.

differentiate between complete cell lysis and selective periplasmic release. Furthermore, in most of these studies cells were harvested from the broth, before they were resuspended in the respective lysis agent. As shown in Table 1, osmotic shock is a commonly used method for extracting periplasmic protein from *E. coli* with high yields [7]. However, sample preparation is cumbersome—harvesting, washing, equilibrating in hypertonic solution, resuspending in distilled water—and thus not possible *in situ* in a bioreactor. Only a few studies on releasing the periplasmic proteins directly to the cultivation broth are available to date [16, 18, 19]. Such a strategy is advantageous, since product can be obtained DC and cell harvesting can be omitted. Furthermore, this approach might be promising in continuous bioprocessing as cells only get leaky but are still viable. Product is continuously harvested and cultivation can be prolonged, as lysis is kept at a minimum. However, to our knowledge, no study that analyzes and compares methods to selectively release periplasmic protein DC without significant cell lysis is available to date.

Here, we performed a systematic and comparative study of how to permeabilize the OM during *E. coli* cultivation processes both in shake flasks and in the controlled environment of a bioreactor. We monitored leakiness and analyzed lysis to be able to distinguish between these two states. To monitor cell integrity, alkaline phosphatase (AP), a marker protein for periplasmic leakiness [23, 24], was measured and cell lysis was monitored using the ß-galactosidase assay [25] and flow cytometry (FCM) [26]. Finally, we combined the addition of a permeabilizing agent with heat treatment, which is known to lead to leakiness of the OM in Gram-negative bacteria [20, 27–29], resulting in a release of nearly 30% of the periplasmic content of the cells while keeping cell lysis at a minimum.

2 Materials and methods

2.1 Chemicals for permeabilizing experiments

We used the following chemicals: TRIS and Pufferan 99.3% (Carl Roth, Karlsruhe, Germany), urea 99.5% (Carl Roth), Triton X-100 (Sigma Aldrich, St. Louis, USA), guanidine hydrochloride 98% (Sigma Aldrich), and benzalkonium chloride (BaCl) 95% (Sigma Aldrich).

2.2 Strain

Escherichia coli BL21(DE3) (Lucigen, Middleton, USA) was used for the periplasmic production of the recombinant model enzyme HRP C1A. The gene encoding HRP was condon optimized from GenScript USA Inc. (NJ, USA) and integrated in a pET39b+ vector (Novagen, San Diego, USA). Translocation of the product to the periplasmic space was accomplished using the DsbA pathway.

2.3 Media

Defined DeLisa minimal medium [30] supplemented with 0.02 g/L kanamycin and different amounts of glucose and lactose were used for all cultivations.

2.4 Shake flask cultivation

Screening experiments to test the impact of different permeabilization agents and their concentration ranges on cell integrity were performed in shake flasks. Precultures were grown in 500 mL of DeLisa medium supplemented with 8 g/L glucose in 2.5-L ultra-yield flasks at 37°C and 160 rpm for 20 h. Then, 5 mL of preculture were aseptically transferred into 500-mL Erlenmeyer flasks containing 45 mL of DeLisa medium supplemented with 8 g/L glucose and 5 g/L lactose to induce recombinant production of HRP at 160 rpm and 30°C. After 4 h, permeabilization agents (Fig. 1 and Supporting Information Table S2) were added as solids and the cultivation was continued overnight.

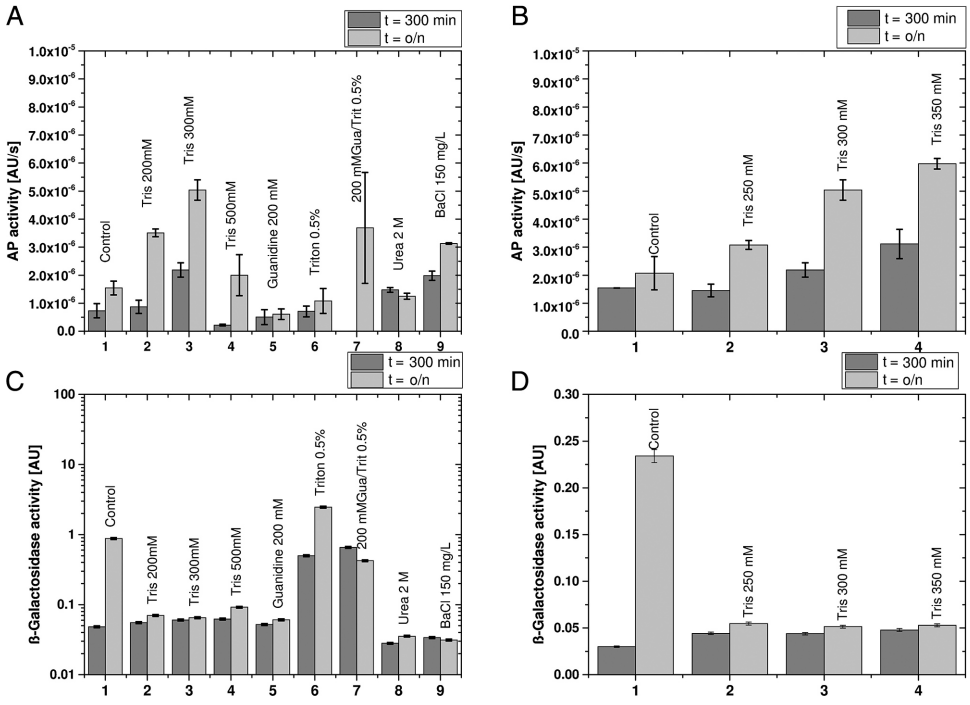

Figure 1. Shake flask experiments for screening of permeabilization agents for their ability to selectively release periplasmic proteins: (A) AP assay indicating periplasmic release for shake flask experiment 1; (B) β-galactosidase assay indication cell lysis for shake flask experiment 1; 1, no addition of agent; 2, TRIS 200 mM; 3, TRIS 300 mM; 4, TRIS 500 mM; 5, guanidine HCl 2 M; 6, Triton 0.5%; 7, 200 mM guanidine HCl + 0.5% Triton; 8, urea 2 M; 9, benzalkonium chloride 150 mg/L; (C) AP assay indicating periplasmic release for shake flask experiment 2; and (D) β-galactosidase assay indication cell lysis for shake flask experiment 1; 1, no addition of agent; 2, 250 mM TRIS; 3, 300 mM TRIS; 4, 350 mM TRIS.

2.5 Bioreactor cultivations

The impact of heat treatment in combination with permeabilization agents on cell integrity was investigated in bioreactor cultivations. The preculture for bioreactor cultivations was identical to the shake flask experiments.

A stainless steel Sartorius Biostat Cplus bioreactor (Sartorius, Göttingen, Germany) with a working volume of 10 L, containing 5000 mL DeLisa medium, supplemented with 20 g/L glucose, was inoculated with 500 mL of preculture. The bioreactor was aerated with a mixture of pressurized air and pure oxygen at 1.5 vvm and agitated constantly at 1000 rpm. Dissolved oxygen was monitored with a fluorescence dissolved oxygen electrode Visiferm DO425 (Hamilton, Reno, NV, USA) and kept above 40% throughout all cultivations by varying the ratio of pressurized air to pure oxygen. pH was monitored with an Easyferm electrode (Hamilton) and maintained constant at pH 7.2 by the addition of NH_4OH (12.5%). Base consumption was determined gravimetrically. CO_2 and O_2 concentrations in the off-gas were monitored by a DASGIP gas analyzer (Eppendorf, Hamburg, Germany). All process parameters were adjusted and logged by the process information management system Lucullus (Biospectra, Schlieren, Switzerland).

The batch phase was conducted at 35°C and yielded a biomass concentration of 8–9 g dry cell weight per liter. After depletion of glucose, visible by a drop in the CO_2 off-gas signal, a fed-batch cultivation to generate biomass was conducted. The feed rate during this phase was adjusted to maintain a constant specific glucose uptake rate ($q_{s,glu}$) of 0.2 g/g/h.

When the dry cell weight reached 25 g/L, the culture was induced by one point addition of IPTG (0.05 mM) or lactose, which was supplied continuously by a mixed feed containing glucose and lactose. Detailed information about the specific uptake rates of glucose and lactose in the different experiments are given in Supporting Information Table S1. After 10 h of induction, periplasmic release was triggered by heat treatment and/or addition of TRIS. Detailed information about the temperature ramps and time points of TRIS addition can be found in the result section in Fig. 2–4.

2.6 Analytics

2.6.1 AP assay

AP, a native periplasmic protein of *E. coli*, serves as a tracer protein for the periplasmic space and was used for the analysis of leakiness [23, 24]. A photometric assay using a 96-well plate was carried out in an *Infinite 200 Pro* plate reader (Tecan, Zürich, Switzerland). One hundred microliters of centrifuged culture broth (5000 *g*, 10 min) were mixed with 100 μL of para-nitrophenylphosphate disodium salt hexahydrate solution (5 g/L) in TRIS buffer at pH 8.5. A kinetic cycle recorded the absorbance at 405 nm for 80 min. Calibration was performed using commercially available AP from bovine intestinal mucosa (Sigma Aldrich) exhibiting 3730 U/mg.

To quantify 100% of periplasmic leakiness, cells were homogenized in an EmusiflexC3 Homogenizer (Avestin, Ottowa, ON, USA) at 1500 bar for five passages. After centrifugation, AP was measured in the supernatant.

2.6.2 β-Galactosidase assay

In shake flask experiments, ß-galactosidase, a native cytoplasmic *E. coli* enzyme, was used for monitoring of cell lysis. Fifty microliters of centrifuged culture broth (5000 *g*, 10 min) were added to 50 μL 2× Assay Buffer (200 mM Na_3PO_4, 2 mM $MgCl_2$, 100 mM ß-mercaptoethanol, and 1.33 mg/mL ONPG). After

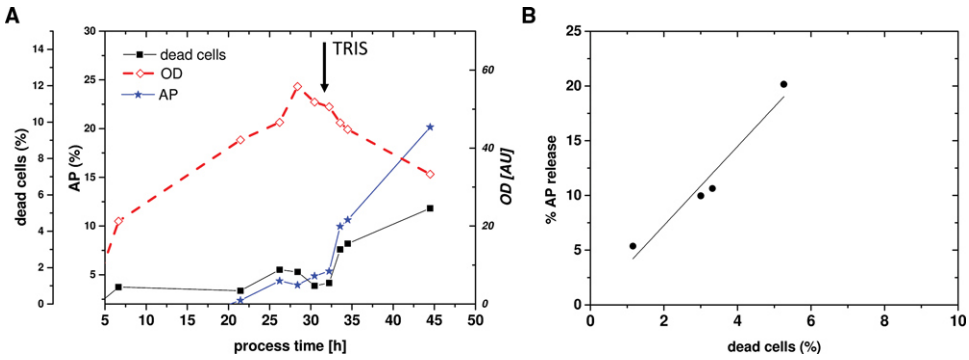

Figure 2. Cultivation 1 for permeabilization of the OM by the addition of 350 mM TRIS: (A) optical density at 600 nm (OD), extracellular AP activity and ratio of dead cells monitored by FCM; (B) scatter plot showing dead cells (*x*-axis) versus periplasmic leakiness (AP release, *y*-axis).

30 min of incubation at 37°C in the plate reader *Infinite 200 Pro* (Tecan, Zürich, Switzerland), the reaction was stopped by the addition of 150 μL 1 M Na_2CO_3 and absorption was measured at 420 nm.

2.6.3 Flow cytometry

FCM was used for lysis monitoring during the cultivations and the permeabilization experiments according to Langemann et al. [26]. We used a CyFlow® Cube 8 flow cytometer (Partec, Münster, Germany). Data were collected using the software CyView Cube 15 and analyzed with the software FCS Express V4 (DeNovo Software, Los Angeles, CA, USA).

3 Results and discussion

The goal of this study was to selectively trigger leakiness of the periplasm by process technological means to release recombinant products during *E. coli* cultivation without cell lysis. We (i) tested the applicability of cell integrity monitoring assays for the used medium and permeabilizing agents, (ii) tested different permeabilization agents regarding their ability to selectively trigger leakiness in shake flask cultivations, (iii) applied the most promising results from shake flask experiments in bioreactor cultivations, (iv) tested the impact of heat treatment on selective periplasmic release, and (v) combined the addition of a permeabilization agent and heat treatment to trigger the release of periplasmic proteins while keeping cell lysis at a minimum.

3.1 Applicability of cell integrity monitoring assays

For cell integrity monitoring, different assays are described in the literature. Extracellular DNA content (e.g. by Nanodrop and Picogreen), the presence of the cytosolic enzyme ß-galactosidase, and FCM measurements can be used to monitor cell lysis. The presence of extracellular AP, a native *E.coli* marker protein for the periplasmic space, can be used to monitor leakiness. An increase in the total extracellular protein content (e.g. by bicinchoninic acid and Bradford assay) can result from both lysis and leakiness.

All assays mentioned above but FCM are photometric assays and thus different matrices can severely affect the results of the assays. Therefore, sensitivity of the assays toward the permeabilizing agents was tested. As FCM relies on another measurement principle where no interactions with permeabilizing agents are expected, this assay was not investigated in this respect. Table 2 shows an overview of the performed experiments to evaluate the sensitivity of the assays toward the permeabilization agents.

Nanodrop showed high interferences with DeLisa medium and was thus not applicable. PicoGreen showed good results for DNA quantification but, as reagents are expensive, this assay was not used in this study. Furthermore, DNA is constantly being digested by released DNases. Without knowledge of digestion rates, DNA quantification results are highly uncertain and cannot be used for proper lysis monitoring.

Beta-galactosidase showed slightly reduced signals in the presence of TRIS and slightly increased signals in the presence of Triton. As this assay is cheap, fast, and robust, it was used to determine cell lysis in shake flask experiments. However, FCM was used for lysis monitoring in bioreactor cultivations.

Minor effects of medium and permeabilization agents were observed for the AP and HRP assays. As HRP needs incorporation of the cofactor heme to be active, we chose the AP assay to monitor integrity of the periplasm.

For total protein determination, the bicinchoninic acid assay was not suitable in combination with DeLisa medium due to high background noise. Bradford measurements, using Coomassie Brilliant Blue G-250, showed only slight increase when TRIS and BaCl were present. However, as the determination of total extracellular protein content does not allow differentiation between leakiness and lysis, we decided to use AP measurements to monitor leakiness and β-galactosidase measurements and FCM to monitor lysis in this study.

3.2 Shake flask screening of permeabilization agents

All shake flasks experiments comprised a phase for biomass generation, an induction phase for recombinant protein production, and a phase for releasing periplasmic proteins by the addition of different permeabilization agents. The agents were chosen according to removability, toxicity and price. The results for periplasmic release, measured by the AP assay, and complete lysis, measured by the β-galactosidase assay, are shown in Fig. 1 and Supporting Information Table S2.

In the first shake flask experiment, the agents TRIS, guanidine HCl, Triton, urea, BaCl, and combinations thereof were tested (Fig. 1A and B). TRIS (300 mM), a combination of guanidine

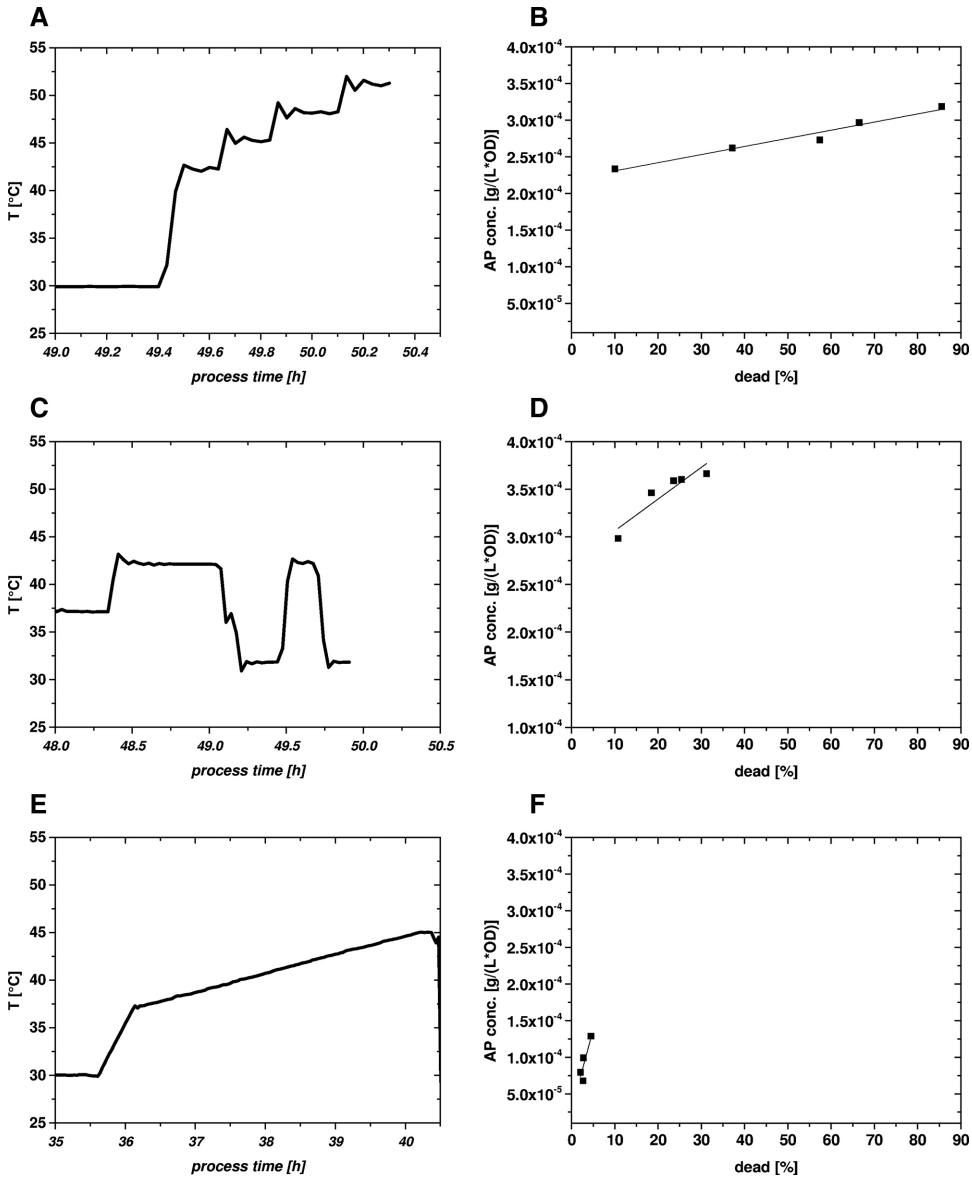

Figure 3. Cultivations 2–4 for permeabilization of the OM by heat treatment; left side: temperature profiles: (A) cultivation 2, stepwise increase (42–51°C); (C) cultivation 3, oscillating temperatures (32–41°C); (E) cultivation 4, ramp (30–42°C); right side: scatter plot showing dead cells (*x*-axis) versus periplasmic leakiness (AP release, *y*-axis); (B) cultivation 2; (D) cultivation 3; and (E) cultivation 4. As in cultivations 2 and 3 lysis was very pronounced, the samples of these cultivations were not homogenized. Therefore, AP values are not given in (%) but as normalized values in [g/(L·OD)].

HCl (200 mM) + Triton (0.5%) and BaCl (150 mg/L) were found to be the most promising candidates regarding periplasmic release (Fig. 1A). Observed lysis in the shake flask where no agent was added (Fig. 1B) was probably a result of substrate depletion as these cultures showed much higher uptake of substrate and faster growth compared to cultures containing permeabilization agent. In fact, in cultures containing permeabilization agent hardly any growth was observed and the cells appeared to be "viable but nonculturable".

As guanidine HCl (200 mM) + Triton (0.5%) led to increased lysis (Fig. 1B) and BaCl is a biocide, we decided to perform periplasmic release with TRIS as this permeabilization agent (i) showed highest periplasmic release, (ii) triggered a low degree of lysis, and (iii) is a cheap chemical that is often used as it is easily removable and nontoxic.

In a second experiment, the impact of different TRIS concentrations on leakiness and lysis was investigated (Fig. 1C and D). TRIS at a final concentration of 350 mM showed the best results regarding periplasmic release with only minor lysis of less than 10% of all cells. Similar results were also found by Irvin and Hancock [15, 31].

3.3 Permeabilization of cells in bioreactor cultivation using 350 mM TRIS

As the addition of 350 mM TRIS was most suitable for permeabilizing the cells with only low levels of lysis in shake flasks, this approach was also tested in the controlled environment of a bioreactor. The cultivation comprised (i) a batch phase, (ii) a fed-batch phase for biomass generation, (iii) an induction phase for recombinant periplasmic protein production, and (iv) a phase for OM permeabilization (cultivation 1; Fig. 2 and Supporting Information Table S1).

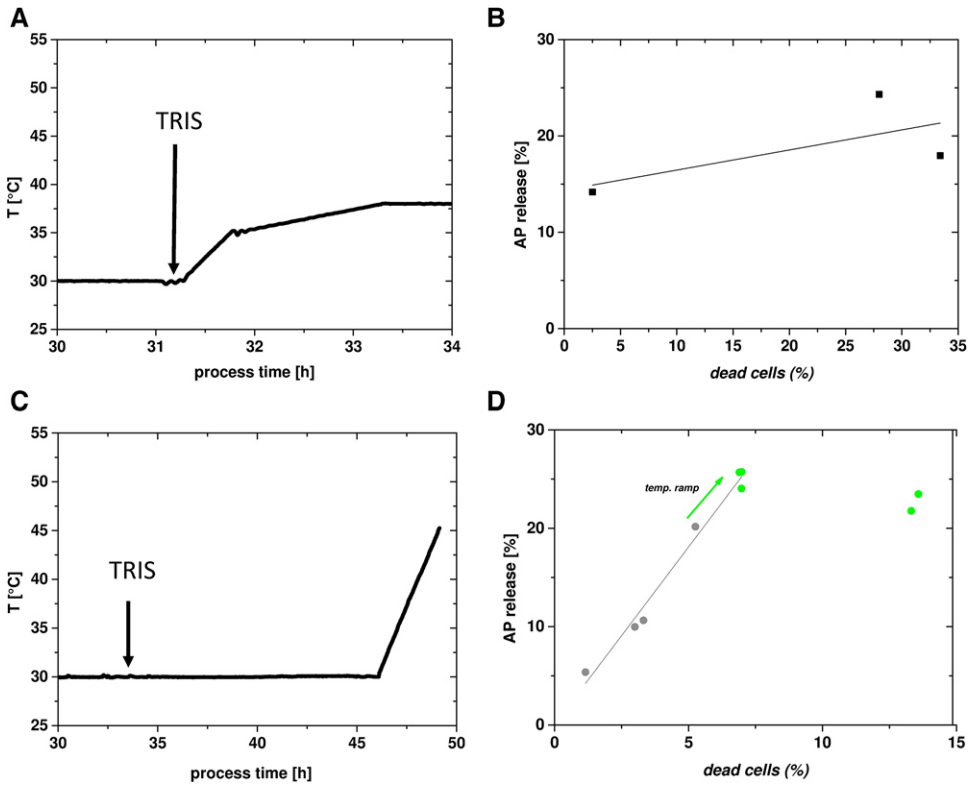

Figure 4. Cultivations 5 and 6 for permeabilization of the OM by combining TRIS addition and heat treatment; left side: temperature profiles and TRIS addition: (A) cultivation 5, (C) cultivation 6; right side: scatter plot showing dead cells (*x*-axis) versus periplasmic leakiness (AP release, *y*-axis), (B) cultivation 5, and (D) cultivation 6.

Table 2. Overview of interferences of cell integrity assays with permeabilization agents

Assay	Control DeLisa	Tris 200 mM	Tris 300 mM	Tris 400 mM	Tris 500 mM	Guanidine 200 mM	Triton 0.5 wt%	Urea 2 M	BaCl 60 mg/L	BaCl 300 mg/L
Nanodrop (DNA)	−	−	−	−	−	−	−	−	−	−
PicoGreen (DNA)	+	+	+	+	+	n/a	n/a	n/a	n/a	n/a
CP marker (β-Gal)	+	+/red	+/red	+/red	+/red	+	+/inc	+	−	+
PP marker (AP)	+/red	+/red	+/red	+/red	+/red	+/red	+/red	+/red	+/red	+/red
PP product (HRP)	+	+/red	+/red	+/red	+/red	+	+	+/inc	+/inc	+/inc
Total protein (bicinchoninic acid)	−	−	−	−	−	−	−	−	−	−
Total protein (Bradford)	+	+/inc	+/inc	+/inc	+/inc	+	−	+	−	+/inc

+, no interference; −, strong interference; red, slightly reduced signal; inc, slightly increased signal; n/a, not available; BaCl, benzalkonium chloride.

Dissolved TRIS stock solution (3.5 M) in deionized water with a pH of 7.8 was added to the bioreactor to a final concentration of 350 mM and cells were subsequently incubated for 12 h. The drop in the OD signal at around 30 h process time is the result of dilution by the addition of TRIS. The further decrease in the OD is the result of dilution by feeding as cells are viable but nonculturable. The same effect of growth inhibition by the addition of TRIS was observed in shake flask experiments. A strong increase in AP release was seen within 1 h after the addition of TRIS, which further increased over the next 12 h. The maximum amount of extracellular protein of about 22% was observed after 12 h of incubation with only about 5% lysis (Fig. 2B). Those results looked promising but still the majority of periplasmic protein was inside the cells. We also tested incubation with TRIS at high basic pH values, but obtained high lysis rates and did not investigate this further (data not shown).

3.4 Permeabilization of cells in bioreactor cultivation using heat treatment

To further increase the selective release of periplasmic proteins, heat treatment was tested. Within this study, we tried three different temperature profiles ranging between 30 and 51°C (Fig. 3).

As in cultivations 2 and 3 lysis was very pronounced, the samples of these cultivations were not homogenized. Therefore, AP values are not given in (%) but as normalized values in (g/(L·OD)) in Fig. 3.

In cultivation 2, we increased the temperature stepwise from 30 to 51°C. However, these conditions were too harsh and resulted not only in leakiness but also in pronounced lysis of almost 40% after the first temperature step. Accompanying release of proteases probably degraded the AP and thus resulted in a

reduced AP signal. Therefore, in cultivation 3 a lower temperature range was tested. Changing the temperature between 32 and 41°C resulted in lower lysis and increased the slope in the AP versus lysis plot.

In cultivation 4, a fast ramp to 37°C with a subsequent linear ramp of 1°/h up to 42°C was applied. This rather mild treatment showed an increase in leakiness up to 7.5% within 4 h while keeping lysis below 5% and thus was the most promising approach. Similar results are shown in [29], where higher temperatures led to a significant increase in cellular lipopolysaccharides in the supernatant when temperature was above 48°C. Nevertheless, in those experiments there was a high degree of cell lysis.

In summary, we found that a rather mild heat treatment using linear ramps increased OM leakiness with only minor cell lysis. However, when temperature exceeded 40°C, pronounced lysis was detected.

3.5 Combination of permeabilizing agents and heat treatment

As treatment with 350 mM TRIS caused leakiness of around 20% and heat treatment with a moderate linear temperature ramp led to 7.5% leakiness, those conditions were combined in cultivations 5 and 6 (Fig. 4). The aim of these cultivations was to achieve a synergetic effect of both applied methods to increase leakiness.

In cultivation 5 (Fig. 4A and B), temperature was increased using a two-step linear gradient right after addition of 350 mM TRIS. After the first temperature ramp, we already detected a high amount of cells lysis of about 25%. After the second ramp, the amount of lysed cells was already close to 35%. Apparently, the addition of 350 mM TRIS and a concomitant increase in temperature was too harsh for the cells.

Thus, in cultivation 6 the cells were incubated for 18 h with TRIS before applying a mild heat treatment (Fig. 4C). The heat treatment in cultivation 6 resembled the temperature profile of cultivation 4 (Fig. 3E) but without the steep ramp in the beginning to give the cells time to adapt. When the temperature was then increased to 38°C, nearly 30% of periplasmic proteins were released and only around 5% of the cells lysed. At higher temperatures, we only found an increase in cell lysis but no significant increase in leakiness. Thus, to trigger leakiness with only minimal lysis, we recommend incubation with 350 mM TRIS for several hours followed by a mild heat treatment up to 38°C.

4 Concluding remarks

In this study, we screened different methods for releasing periplasmic products from recombinant *E. coli* to the cultivation supernatant to simplify downstream processing. By the developed strategy, about 30% of the periplasmic content containing the target product was released without significant cell lysis (<5%). As cells are still viable after product release, this approach combined with suitable induction strategies might be an interesting method for continuous bioprocessing. In summary, this study represents a comparative study of different strategies to trigger *E. coli* leakiness and describes a solid basis for further experiments in this field.

Practical application

Recovery of recombinant product from *E. coli* is usually performed by harvesting the cells and subsequent cell disruption. This causes release of not only target protein but also a vast amount of impurities. In this work, we describe a comparative approach to selectively release periplasmic product from *E. coli* during bioreactor cultivation without triggering cell lysis. This strategy leads to less impurities in the crude product, simplifies the downstream process, and might pave the way for continuous bioprocessing.

The authors have declared no conflict of interest.

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CONCLUSIONS

Following scientific questions could be clarified during the course of this thesis:

1st question: Can the VCC be measured reliably online during the process using FCM and an impedance spectroscopic based online probe in the α-dispersion range?

FCM is a very appropriate method for validation of dead and living cell in atline/offline mode and was therefore used for later measurements of the periplasmic release. A online probe based on EIS was established and validated during the batch and fedbatch cultivation with *BL21(DE)3*. The developed method is easyly applicable and has high future potential in food-industry or for other microbial cultures in online or inline mode.

2nd question: Can the knowledge found in question 1 be used to find a detergent for selectively release of a model periplasmic protein - like alkaline phosphatase - without killing the host cell during the release process?

Different detergents known for their ability to destabilize the OM were tested. An optimum for *BL21(DE)3* in terms of measurement of alkaline phosphatase activity in the supernatant was found for 350 mM Tris. Lysis degree was measured using β-galactosidase assays for easy application and high throughput reasons. This easy to use method with a very cheap reagent may be used for low value - high volume products, since the down stream costs are often the major investment in these products.

3rd question: Is it possible to apply the best detergent in the optimized concentration range during a fermentation process? Can synergistic effects be achieved using heat ramps and shocks during the release experiment?

Different settings were tested for 350 mM Tris with/without heat experiments. It is found that 30 % in maximum can be released during incubation with Tris over night and subsequent heat shock in situ without significant cell death. This possible method may be used for continuous product release in a fedbatch approach and even be a method to be used in contentious processing.

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Periplasmatischer Produktrelease in *E. coli* mittels verschiedener Chemikalien
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Engineering in Life Sciences

Publikation - 11/2016 Erstautor - A novel application for low frequency electrochemical impedance spectroscopy as online process monitoring tool for viable cell concentrations

Nutzen von Impedanzspektroskopie zur Bestimmung der Viabilität in bakteriellen Kultivierungen
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Publikation - 9/2016 Mitautor - The Chemical Capacitance as a Fingerprint of Defect Chemistry in Mixed Conducting Oxides

Review über die Änderungen der chemischen Kapazität in verschiedenen oxidischen Materialien
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Publikation - 11/2016 Erstautor - The Effect of Nominal Acceptor and Donor Doping on Oxygen Vacancy Concentrations in PZT

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