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"Process Development for Industrial Scale Bacterial Ghost Production"

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6.1 Introduction

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Objectives

Bacterial Ghosts (BGs) are empty bacterial envelopes. They are produced from Gram-negative bacteria by controlled expression of cloned bacteriophage φX174 lysis gene E. The outstanding feature of the E-lysis process is that the bacterial morphology is maintained since protein E does not exhibit any enzymatic activity. Instead, it induces formation of a trans-membrane tunnel structure in the bacterial envelope through which the cytoplasmic contents is expelled. The remaining structurally intact shell is called Bacterial Ghost. BGs are unique structures as they exactly resemble their viable counterparts but are no longer reproducible and devoid of genetic information. With the BGs antigenic structures such as e.g. LPS, pili or membrane proteins are conserved. Where necessary, the BG structure can also be augmented with foreign target antigens (TAs) prior to E-lysis. BGs have intrinsic adjuvant properties and the ability to target primary antigen-presenting cells (APCs). Due to their outstanding properties BGs have been suggested as candidate vaccines or drug delivery vehicles. Immunogenicity of BGs for both native and foreign TAs has been shown in animal models. Targeting of professional APCs and tumor cells with drug-loaded BGs leading to BG-internalization and subsequent drug-release was demonstrated as well.

In order to take the next step towards an industrial BG product it is important to establish a suitable production process. Such process should be cost-efficient, comparably fast and as incomplete as possible. BGs are intended for application as active pharmaceutical ingredients (APIs) which means that the production process must be setup to meet the standards of good manufacturing practice (GMP).

The main findings of this thesis are concluded in chapters 3 to 6. Those chapters all have the underlying structure of discrete manuscripts featuring individual abstracts and brief introductions as well as sections for materials and methods, results and a discussion.

Production of DNA-free *Shigella flexneri* 2a Bacterial Ghosts

BGs derived from different pathogens have been suggested as candidate vaccines and tested successfully for immunization in animals. In some cases BGs even proved to be superior to
inactivated bacterins providing sterile immunity. Acknowledging the benefits that animal
BG candidate vaccines entail the concept should be extended to human vaccines. One major
health issue in developing countries is shigellosis which is caused predominantly by Shigella
flexneri serotype 2a (Sf2a). Development of an efficient BG candidate vaccine against Sf2a
would be an approach to help contain shigellosis.

In order to conduct pre-clinical studies and subsequently clinical trials sufficient amounts
of a safe BG candidate vaccine would be required. A suitable production process in complex
medium should meet a number of requirements: a mandatory E-lysis efficiency of 99.9%,
non-denaturating process conditions to maintain antigenicity and a fully inactivated prod-
uct. In addition it would be desirable to obtain a DNA-free product. It should be shown
that E-lysis is working satisfyingly in Sf2a. The additional use of staphylococcal nuclease A
(SNUC) should provide further reduction of viability and a high degree of DNA degradation
as determined by real-time PCR. At the end of the production process the lyophilized BG
product should be bioburden free.

**Bacterial Ghost production: β-propiolactone inactivation of non-lysed bacteria**

The chemical β-propiolactone (BPL) has been described as a suitable agent for virus-
inactivation in medicinal products such as vaccines. It has further been suggested for inac-
tivation of whole-cell vaccines. As BG candidate vaccines have to be fully inactivated at the
end of the production process BPL is also used for this purpose. BPL alkylates DNA and
prevents DNA transcription and replication.

The amount of BPL required for product inactivation should be proportional to the total
amount of DNA present. In case of BG production the vast majority of DNA after E-lysis
is present as free DNA in the supernatant. Implementation of tangential flow filtration into
downstream processing for concentration and washing of the BG product should drastically
reduce the amount of free DNA. This effect should be detectable by simple photometric
DNA measurements in the supernatant. Postponing BPL treatment to late in the down-
stream procedure should therefore help to minimize the amount of BPL required for total
inactivation. Once a minimal amount of BPL is determined for a given concentration of
surviving cells the results should be extendable given that the concentration of viable cells
and the concentration of residual free DNA can be reliably estimated.

**Real-time assessment of E-lysis performance in Escherichia coli through flow cytometry**

Flow cytometry (FCM) is a well-established tool for determination of single cell-properties
Objectives

in a population. Assays for various parameters and especially viability have been developed. Assessment of E-lysis by FCM has been described combining information gathered from scatter and fluorescence signals using a membrane potential-sensitive dye.

Adapting the described procedure with a state of the art flow cytometer should be feasible for small and large scale BG production. Introducing a second fluorescent dye for labeling of cell membranes should enable the definition of a gate identifying cellular particles and exclude non-cellular background. By this measure the quality of FCM data obtained while following E-lysis from should improve drastically. Furthermore, using FCM during BG fermentations should provide deeper insight into the kinetics of E-lysis in *Escherichia coli* since FCM results are generated in quasi real-time.

**High-density fermentation of *Escherichia coli Nissle 1917* for Bacterial Ghost production**

Utilizing the features of probiotic *Escherichia coli* strain Nissle 1917 (EcN) it would be possible to provide a potent range of BG products. Due to the immunostimulant properties of EcN the corresponding BGs are expected to be usable as adjuvants, as supplements inducing a non-specific immune boost or as drug delivery vehicles. Production of BGs has been described as a low density process in complex medium while harvesting of the BG product was done through a separator.

Industrial EcN BG products require a competitive high-quality production process. The change to a minimal culture medium should allow for fermentation to much higher cell densities and is also anticipated to clear up doubts arising from potential contaminations with animal-protein in complex media. Using batch and fed-batch fermentation techniques it should be possible to increase cell densities before E-lysis induction without losing E-lysis efficiency. Incorporation of tangential flow filtration (TFF) into the process should help to improve product quality as TFF is conducted in a sterile closed system and also avoids pelletization of the product. Integrating findings from chapters 4 should facilitate the correct estimation of the required amount of BPL for total product inactivation. Applying the FCM method established in chapter 5 during the production process should provide a reliable tool for real-time assessment of process performance.
Summary

This thesis ‘Process Development for Industrial Scale Bacterial Ghost Production’ describes the development of a BG production process from a low density batch process in complex medium towards a high density fed-batch approach in minimal medium. Specific changes in the fermentation and harvesting procedures allowed to reach higher yields while at the same time product quality was improved. Real-time parameters for the evaluation of process performance were introduced to make the production more efficient.

Production of DNA-free \textit{Shigella flexneri} 2a Bacterial Ghosts

In a first step a stable low density production process for \textit{Shigella flexneri} 2a BGs was presented. This process combined the features of E-lysis and staphylococcal nuclease A (SNUC). The latter effected enhanced reduction of viability and moreover significant degradation of DNA in the product. In a consistency study of five production runs performed under identical conditions a mean E-lysis efficiency of 99.94% was obtained. After 90 min of E-lysis and another 180 min of SNUC activity the viability of the cultures was overall reduced by an average of almost six decimal powers. Furthermore, it could be shown that the DNA contents in the product was reduced to the detection limit of real-time PCR by the nuclease indicating the product was essentially DNA-free. Chemical inactivation with 0.75%/per thousand zero (v/v) \(\beta\)-propiolactone before harvesting by mechanical separation yielded a bioburden-free product which was verified by sterility tests before and after lyophilization.

Bacterial Ghost production: \(\beta\)-propiolactone inactivation of non-lysed bacteria

The harvesting procedure for BG production was changed from mechanical separation to tangential flow filtration. The culture broth of low density Sf2a BG fermentations (cell densities \(\leq 10^9\) cells per ml) was concentrated by a factor 10 before inactivation thereby reducing the total amount of free DNA in the suspension by 90%. Screening experiments with Sf2a indicated that by this measure the required amount of BPL could also be reduced by 90% to now 0.075%/v/v. Results were confirmed for low density fermentations of
E. coli BGs. The application temperature is determined by the respective temperature of E-lysis induction: 44°C for Sf2a and 42°C for E. coli. For medium density fermentations of EcN BGs with ten-fold higher cell densities (≤ 10^{10} cells per ml) where dia-filtration was incorporated into the E-lysis phase the concentration of free DNA could be reduced by at least 30% even before concentration of the broth. This reflected in the amount of required BPL which was only 0.5% (v/v\textsubscript{f}). This showed that in case the amount of residual free DNA in the concentrate can be reliably estimated the required amount of BPL for total inactivation of BG products can be calculated as a function of cell density.

Real-time assessment of E-lysis performance in *Escherichia coli* through flow cytometry

A flow cytometry-based method for the evaluation of E-lysis in *E. coli* was presented. Based on a previously suggested assay an improved method was established. Utilizing the change in translucency caused by E-lysis which impacts the cells forward scatter signal and combining this with viability information obtained from the fluorescence signal of membrane potential-sensitive dye DiBac\(_4\)(3) gave good differentiation between live, whole dead and E-lysed bacteria. Using red-fluorescent dye RH414 as a cell membrane marker enhanced the quality of FCM data as non-cellular noise could be effectively excluded. The described method allowed to use FCM as an eligible quality criterion for BG production in quasi real-time.

High-density fermentation of *Escherichia coli* Nissle 1917 for Bacterial Ghost production

Using a minimal medium for batch production of *Escherichia coli* Nissle 1917 BGs the productivity could be increased by the factor 10 (medium cell density) as compared to the standard low density fermentation without losing E-lysis efficiency. Good conformance between FCM and conventional cfu data was shown. For this medium density approach using tangential flow filtration was of great value. Due to expulsion of the cytoplasm at elevated cell densities the culture broth viscosity changed significantly and foaming issues occurred. Incorporating a first dia-filtration step into E-lysis phase resolved those problems and also had the beneficial side-effect of removing free DNA from the broth mentioned above. Over a total of nine production cycles a high degree of reproducibility was shown for medium density production of EcN BGs. Average cell densities were 7.35 · 10^{9} live EcN per ml while the average E-lysis efficiency was 99.78%. BPL treatment with 0.5% (v/v\textsubscript{f}) was sufficient in all cases yielding a bioburden free product after lyophilization.

In addition, a high-density fed-batch process in minimal medium was presented. Initial
data generated showed that it is possible to further increase cell densities by at least a factor 10 (average cell density: $7.06 \cdot 10^{10}$) with respect to the medium density process. It was evident that feeding has to be maintained into E-lysis phase, the feed profile should be decelerated exponentially. With an adequate feeding strategy after E-lysis induction lysis efficiencies $> 99.8\%$ were reached.
Zielsetzung


Um BGs im industriellen Maßstab herstellen zu können bedarf es eines geeigneten Herstellungsprozesses. Ein solcher Prozess sollte kosteneffizient, vergleichweise schnell und möglichst unkompliziert sein. BGs sind für den Gebrauch als medizinische Wirkstoffe vorgesehen (engl.: active pharmaceutical ingredient, API) und müssen daher gemäß den Vorschriften der guten Herstellungspraxis (engl.: good manufacturing practice, GMP) produziert werden.

Process Development for Industrial Scale BG Production

Herstellung von DNA-freien *Shigella flexneri* 2a Bacterial Ghosts


**Bacterial Ghost Produktion: Inaktivierung von nicht-lysierten Bakterien mit β-Propiolacton**

Die Chemikalie β-Propiolacton (BPL) wurde bereits früh als Reagenz zur Virus-Inaktivierung von medizinischen Produkten vorgeschlagen. Darüber hinaus wird sie auch zur Abtötung von Ganzzell-Impfstoffen verwendet. Da auch BG Impfstoffkandidaten am Ende des Herstellungsprozesses komplett inaktiviert werden müssen, wird ebenfalls BPL verwendet. BPL verhindert die Transkription und Replikation von DNA.

Die Menge an BPL, die für die Inaktivierung nötig ist, sollte daher von der Menge der vorhandenen DNA abhängen. Im Fall von BG Produkten liegt DNA nach der E-Lye beinahe ausschließlich frei im Medium vor. Das Einbinden von Konzentrations- und Waschschritten mittels Querstromfiltration in den Ernteprozess für das BG Produkt sollte die Menge der freien DNA im Produkt drastisch verringern. Dieser Effekt soll durch photometrische Bestimmung der DNA-Konzentration im Überstand der Produktsuspension nachgewiesen werden. Indem die Zugabe von BPL soweit es geht an das Ende des Ernteprozesses verschoben wird, sollte dementsprechend auch die benötigte Menge an BPL zur Inaktivierung reduziert werden können. Nachdem eine minimale Menge an BPL ermittelt wurde, die zur
Zielsetzung

Inaktivierung einer bestimmten Konzentration überlebender Bakterien benötigt wird, sollten diese Ergebnisse übertragbar sein, solange die Menge der überlebenden Zellen bekannt ist und zudem abgeschätzt werden kann, zu welchem Grad die Menge der DNA in der Produktsuspension reduziert werden konnte.

Echtzeit-Bewertung des E-Lyse Prozesses in *Escherichia coli* mittels Durchflusszytometrie


Hochdichte-Fermentation von *Escherichia coli* Nissle 1917 zur Bacterial Ghost Produktion

Mit dem probitischen *Escherichia coli* Stamm Nissle 1917 (EcN) ließe sich eine Reihe vielversprechender BG-Produkte herstellen. Da EcN immunstimulierende Eigenschaften hat, sollten EcN BGs als Adjuvansien, als immunstimulierende Nahrungsergänzung oder als Transportvehikel für Medikamente verwendet werden können. Die Produktion von BGs ist bislang als Fermentation in komplexem Medium mit geringen Zelldichten bekannt. Dabei wird das Produkt mittels Durchflusszentrifugation in einem Separator geerntet.

Zusammenfassung


**Herstellung von DNA-freien *Shigella flexneri* 2a Bacterial Ghosts**


**Bacterial Ghost Produktion: Inaktivierung von nicht-lysierten Bakterien mit β-Propiolacton**

In der Ernteprozedur für BG-Produkte wurde in der Folge die mechanischen Separation durch Querstromfiltration (TFF) ersetzt. Die Kulturbüre von Sf2a BG Fermentationen mit niedriger Zellausbeute (≤ 10⁹ Zellen pro ml) wurden vor dem Inaktivierungsschritt zehnfach
aufkonzentriert, dabei wurde die absolute Menge an freier DNA in der Suspension um 90\% reduziert. Rasterexperimente mit Sf2a zeigten, dass die Menge an benötigtem BPL durch diese Maßnahme ebenfalls um den Faktor zehn auf nurmehr 0.075\% (v/v) reduziert werden konnte. Diese Ergebnisse wurden für BGs auf Fermentationen mit *E. coli* bestätigt. Die Anwendungstemperatur entsprach jeweils der Temperatur zur Induktion der E-Lyse: 44°C für Sf2a und 42°C für *E. coli*. Die Einbindung eines Diafiltrationsschrittes in die E-Lyse Phase bei Fermentationen von EcN BGs mit mittleren Zelldichten (\( \leq 10^{10} \) Zellen pro ml) führte zu einer DNA-Abreicherung von mindestens 30\% noch vor der Aufkonzentrierung der Kulturbrühe. Dies spiegelte sich direkt im BPL-Bedarf wider, der nur 0.50\% (v/v) betrug. So konnte gezeigt werden, dass die benötigte Menge an BPL aus der Zelldichte berechnet werden kann, solange die verbleibende Menge an freier DNA zuverlässig abgeschätzt werden kann.

**Echtzeit-Bewertung des E-Lyse Prozesses in *Escherichia coli* mittels Durchflusszytometrie**


**Hochdichte-Fermentation von *Escherichia coli* Nissle 1917 zur Bacterial Ghost Produktion**

Durch die Verwendung eines definierten Mediums zur Herstellung von *E. coli* Nissle 1917 BGs konnte die Produktivität des Batch-Prozesses im Vergleich zu den Fermentationen mit niedrigen Zellausbeuten um den Faktor 10 gesteigert werden (mittlere Zelldichten), ohne dass die E-Lyseffizienz abnahm. Eine gute Übereinstimmung zwischen Zellzahlbestimmungen durch FCM und herkömmlich ermittelten Zellzahlen mit der Methode der Kolonie-bildenden Einheiten (KBE) wurde gezeigt. Bei der Fermentation mit mittleren Zelldichten war die
Zusammenfassung

Anwendung von Querstromfiltration von großem Wert. Da durch das Ausstoßen des Zytoplasma bei höheren Zeldichten die Viskosität des Medium ansteigt, kam es zu verstärkter Schaumbildung. Das Einbinden eines ersten Diafiltrationsschrittes in die E-Lyse Phase half, diesem Effekt entgegen zu wirken und bewirkte als Nebeneffekt auch die bereits erwähnte Abreicherung freier DNA aus der Kulturbrühe. Die gute Konsistenz des Batch-Prozesses wurde in insgesamt neun Läufen gezeigt. Die durchschnittliche Zelldichte vor der E-Lyse war $7.35 \cdot 10^9$ Zellen pro ml, die mittlere E-Lyseeffizienz lag bei 99.78%. Die Produktinaktivierung mit 0.50‰ (v/v) BPL war in allen Fällen ausreichend und lieferte ein keimfreies Produkt nach der Gefriertrocknung.

1 Introduction

This chapter gives an overview on the major concepts and applied techniques underlying this work. The different topics will be presented consecutively apriori and put into context in chapters 3 to 6.

1.1 Bacterial Ghosts

Detailed reviews about Bacterial Ghosts, the involved genetic and microbiological principles and their applications have recently been published by Walcher et al. 2004 [47], Paukner et al. 2006 [38], Riedmann et al. 2007 [43], Mayr et al. 2005 and 2008 [35,36], Lubitz et al. 2009 [30], Kudela et al. 2010 [27] and Langemann et al. 2010 [28]. The latter review is provided in this chapter.

1.1.1 Background

Bacterial Ghosts (BGs) are empty non-living cell envelopes obtained from Gram-negative bacteria. BGs are produced by controlled expression of the single cloned bacteriophage ϕX174 lysis gene E [12,53]. The gene E product - protein E - is a 91-aa polypeptide [2,41] lacking any known enzymatic function [10,34]. Protein E is a membrane protein which is able to oligomerize and form a discrete membrane-spanning tunnel structure in Escherichia coli. Extensive studies of the structure of protein E [3,5], the biochemical characteristics of protein-E-mediated lysis [50] and evaluation of electron microscopy studies of E-lysed cells [51] led to an understanding of BG formation.

Protein E integrates into the membrane and targets the cells division site where it oligomerizes and induces fusion of the inner and outer membrane forming a discrete E-lysis tunnel [44,52]. The driving force for this membrane fusion is the osmotic pressure difference between the cytoplasm and the surrounding medium [52]. It could be shown that during E-lysis all cytoplasmic contents is expelled while the periplasmic space is sealed and remains as an enclosed space [50,51].
A lot of evidence has been gathered that E-lysis is highly dependent on the physiological state of the host cells [4] and is tightly linked to mechanisms involved in cell division [31, 48]. Stationary phase cells will not respond to E-lysis induction until a growth impulse (e.g. fresh medium) is provided [4]. For an individual cell E-lysis becomes independent from translation and transcription within one minute after effective induction of gene $E$ expression; the onset of lysis happens within the first 10 min [49]. The timeline of E-lysis according to Witte et al. is shown in figure 1.1.

In an exponentially growing population of cells E-lysis effects at least 99.5 and in many cases more than 99.9% of all cells within 120 min after lysis induction (LI). This percentage is referred to as (E-)lysis efficiency (LE) and represents the ratio of BGs and total living cell counts at LI.

Since E-mediated lysis effectively kills the host cell expression of gene $E$ has to be repressed tightly during bacterial growth. Different promoter/operator systems have been used to control E-lysis including $lac\ PO-lacI\beta$ and $\lambda p_r/\lambda p_n\cdot cI857$. For recent BG production a modified temperature-inducible $\lambda p_r\cdot cI857$ system is used allowing for fermentation at 36 or 39°C [20, 21], respectively. LI is enforced by a temperature shift to 42 or 44°C.

BGs have been derived from different Gram-negative strains: i.e. numerous $E.\ coli$ strains, Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Haemophilus influenzae, Helicobacter pylori, Klebsiella pneumoniae, Mannheimia haemolytica, Pasteurella multocida, Pectobacterium cypripedii, Pseudomonas aeruginosa, Pseudomonas putida,Ralstonia eu-
tropha, Salmonella enteritidis, Salmonella typhimurium, Shigella flexneri 2a, Vibrio cholerae, and others establishing confidence that the technology applies in principle for all Gram-negative bacteria.

1.1.2 Applications of BGs

E-lysis happens through a discrete tunnel structure and leaves behind empty but structurally intact cell envelopes. All morphological properties and surface structures remain unaltered. At the same time BGs are devoid of any cytoplasmic residues [50]; most importantly residual DNA. This combination makes BGs promising candidates for a new generation of potent and safe vaccines. With the cell morphology also most antigenic properties are maintained enabling BGs to trigger both cellular and humoral immune response. In addition, BGs feature intrinsic adjuvant properties [43]. Using recombinant DNA technology BGs may also be loaded with foreign protein or DNA antigens. Target antigens (TAs) can be presented both on the inner and outer membrane (IM vs. OM) [15, 46]. TAs that have been excreted to the periplasmic space before E-lysis are retained during BG formation. Presenting foreign TAs in BGs is summarized in a schematic drawing in the BG review, section 1.1.3 [28].

Numerous animal models have been used to investigate immunization against pathogenic Gram-negative bacteria by BGs. For example, vaccination of swine via mucosal application of A. pleuropneumoniae BGs induced sterile immunity against lethal challenge with the pathogen as well as cross-protection against other serotypes [13, 14, 16]. Several other studies for the use of BGs as candidate vaccines have been published and reviewed [17, 18, 32, 33].

A second important field of application is the use of BGs as carrier vehicles. Due to their surface properties BGs are able to target specific tissues, particularly antigen-presenting cells. The inner lumen of BGs can be loaded with biologically active substances (drugs) and delivered to e.g. carcinoma cells [27]. DNA (i.e. minicircles) carrying the lac operator site can be attached to membrane-anchored lacI repressor molecules [19]. The use of BGs as delivery vehicles has been reviewed recently [11, 35, 38].

1.1.3 The Bacterial Ghost platform system - Production and applications
The bacterial ghost platform system
Production and applications

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Key words: E-mediated lysis; bacterial ghosts; vaccines; drug carrier; fermentation; bacterial inactivation; tumor treatment; white biotechnology

Abbreviations: BG, bacterial ghost; IM, inner membrane; OM, outer membrane; PPS, periplasmic space; cfu, colony forming units; dO2, dissolved oxygen; TFF, tangential flow filtration; BPL, β-propiolactone; dH2O, de-ionized water; OD600, optical density at 600 nm; FSC, forward scatter; FL1, fluorescence signal 1; IPTG, isopropyl β-D-thiogalactopyranoside; App, A. pleuropneumoniae; PAMP, pathogen-associated molecular pattern; LPS, lipopolysaccharide; MPL, monophosphoryl lipid A; TLR, toll-like receptor; AG, antigen; CPS, cytoplasmic space; DOX, doxorubicin; Caco-2, colorectal adenocarcinoma; DC, dendritic cell; pSIP, self-immobilizing plasmid; GFP, green fluorescent protein; mc, minicircle; APC, antigen-presenting cell; ADH, alcohol dehydrogenase; IL, ionic liquid

Introduction

Bacterial ghosts (BGs) are envelopes from Gram-negative bacteria which have been produced by controlled expression of the cloned lysis gene E. The essential role of gene E in the lysis of Escherichia coli after infection with bacteriophage ϕX174 was discovered in 1966.1 More than 16 years later, when genetic engineering had been developed, it could be shown that its sole expression after cloning is sufficient to cause subsequent lysis of E. coli.2,3 E was the first lethal gene for bacteria which could be silenced on plasmids. When established in non-host range bacteria of the phage expression of E converts Gram-negative bacteria into BGs whereas Gram-positive bacteria are killed without lysis. Gene E codes for a 91-aa polypeptide,4,5 which, in contrast to lytic proteins from other phages, has no inherent enzymatic function.6 E represents a membrane protein with the ability to oligomerize into a transmembrane tunnel structure.8,9 Analysis of the primary structure of protein E revealed a hydrophobic region at its N-terminal end suggesting a cotranslational integration into the cytoplasmic membrane of E. coli.10 The observations that stationary phase host cells do not respond to E-lysis induction but lyse upon provision of fresh medium and other findings such as the inhibitory effect of non-physiological pH-values on the E-lysis process, indicate that E-mediated lysis is dependent on the growth phase of the host cells and its autolytic system.9,10,13

Analysis of the hydropathicity regions of protein E indicated an E-specific lysis tunnel spanning the inner (IM) and outer membrane (OM) which most probably is located at membrane adhesion sites within the host cell.10 E-mediated lysis forms the BG by releasing all cytoplasmic content to the environment while periplasmic components remained associated with the empty cell envelope.1 The collapse of the bacterial membrane potential precedes the onset of E-lysis.12 When the E-lysed E. coli were viewed by high-magnification scanning and transmission electron microscopy, the E-specific lysis tunnel was observed (Fig. 1A).

The Bacterial Ghost (BG) platform technology is an innovative system for vaccine, drug or active substance delivery and for technical applications in white biotechnology. BGs are cell envelopes derived from Gram-negative bacteria. BGs are devoid of all cytoplasmic content but have a preserved cellular morphology including all cell surface structures. Using BGs as delivery vehicles for subunit or DNA-vaccines the particle structure and surface properties of BGs are targeting the carrier itself to primary antigen-presenting cells. Furthermore, BGs exhibit intrinsic adjuvant properties and trigger an enhanced humoral and cellular immune response to the target antigen. Multiple antigens of the native BG envelope and recombinant protein or DNA antigens can be combined in a single type of BG. Antigens can be presented on the inner or outer membrane of the BG as well as in the periplasm that is sealed during BG formation. Drugs or supplements can also be loaded to the internal lumen or periplasmic space of the carrier. BGs are produced by batch fermentation with subsequent product recovery and purification via tangential flow filtration. For safety reasons all residual bacterial DNA is inactivated during the BG production process by the use of staphylococcal nuclease A and/or the treatment with β-propiolactone. After purification BGs can be stored long-term at ambient room temperature as lyophilized product. The production cycle from inoculation of the pre-culture to the purified BG concentrate ready for lyophilization does not take longer than a day and thus meets modern criteria of rapid vaccine production rather than keeping large stocks of vaccines. The broad spectrum of possible applications in combination with the comparatively low production costs make the BG platform technology a safe and sophisticated product for the targeted delivery of vaccines and active agents as well as carrier of immobilized enzymes for applications in white biotechnology.
three-phase model: (1) integration of protein E into the IM with the C-terminus facing the cytoplasm; (2) conformational change of protein E translocating the C-terminal domain to the PPS accompanied by oligomerization and targeting of the division initiation complex via lateral diffusion; (3) fusion of IM and OM at membrane adhesion sites induced by exposition of the C-terminus of protein E to the cell surface. This model implies that the lysis tunnel is not solely bordered by protein E oligomers but its formation requires protein E-triggered fusion of the inner and outer membrane.23 A schematic drawing of this model is shown in Figure 2B.

Upon discovery of the remarkable features of protein E-mediated lysis in *E. coli*, the principle of E-lysis could be shown with other Gram-negative bacteria24,25 but not for Gram-positive bacteria.26 So far, BGs of numerous Gram-negative strains (different *E. coli* strains, *Salmonella typhimurium*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Bordetella bronchiophila*, *Helicobacter pylori*, *Vibrio cholerae*, *Actinobacillus pleuropneumoniae*, *Haemophilus influenzae*, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Ralstonia eutropha*, *Pectobacterium cypripedii* and others) have been generated successfully. This suggests that the BG platform might be extended to any Gram-negative bacterium.25 The idea of utilizing BGs derived from different Gram-negative bacteria as candidate vaccines emerged due to the demand for both potent and safe new vaccines.27-31 The BG system offers many advantages over traditional vaccination techniques including targeting and the intrinsic adjuvant properties of the BG particles. In addition, recombinant DNA technology facilitates the development of multivalent protein or DNA vaccines. Another great feature of BGs is the fact that no denaturing effects occur during E-lysis and hence all antigenic determinants are preserved throughout BG generation. The use of BGs as candidate vaccines and advanced drug carriers can be found in several recent reviews.24,27,29,32-36

**BG—Production Process**

Initial cloning and expression studies with gene E used the inducible *lac* promoter/operator system with an overexpression of the *lac* repressor gene (*lac* PO-*lacI*)2,3. Later the temperature-sensitive λ-system (*λ* <sub>pL</sub>/*λ* <sub>pR</sub>-*ci857*) has proven to be more suitable for quick and efficient lysis without the need of any addition of chemical inducers.18 Since the *λ* repressor *ci857* shows incipient expression of downstream gene *E* at temperatures above 30°C, the temperature sensitivity of the system was optimized to meet more favorable fermentation temperatures of 35°C or higher. Mutations in the OR2 operator region of the *λ* <sub>pR</sub> promoter resulted in tight repression of downstream genes up to 36°C and 39°C, respectively.27,36 These temperature-inducible E expression cassettes are

**Figure 1.** (A) Lysis tunnel formation and expulsion of the cytoplasmic contents—reproduced from Ebensen et al.32 (B) Lysis tunnel formation, accompanied by the fusion of IM and OM (arrow)—reproduced from Witte et al.18
Figure 2. (A) Different methods for AG presentation in the BG envelope complex—BG themselves carry native AG (LPS, OMP, IMP, TCP, flagella, pili)—TA may be presented on the cell surface via fusion with OmpA—the PPS can be loaded with TA via MBP-SbsA-fusion proteins (1), by fusion of the TA with MBP (2) or as sole TA using the gene III signal sequence (3) Protein TA may be incorporated into the IM via E', L' or E'/L'-anchoring, biotinylated AG can be attached to E'-FXa-StrpA membrane anchors, DNA carrying the lac operator site can be attached to L'-anchored lacI repressor molecules—TA fused with SbsA-/SbsB proteins form S-layers in the PPS. (B) Model of lysis tunnel formation according to Schön et al.23
widely used in current BG production processes since they are robust enough to allow fermentation of the bacterial culture at 35°C and induction of protein E-mediated lysis at 42 or 44°C.

In standard fermentations of various bacteria the quality criterion for a successful E-lysis process is a BG formation of at least 99.9% of the bacterial culture within a time window of 2 h. Depending on the host organism E-lysis efficiencies of more than 99.9% and higher can be achieved in this time frame. In Figure 3 the time-point of E-lysis induction is defined as time-point zero (0 min) with the preceding growth phase denoted in negative minutes.

BG production has been established in fermentation volumes up to 20 l using Labfors-3 and Techfors-S fermenters (Infors HT, Bottmingen, CH). Staring with a pre-culture that is growing exponentially, the production fermenter is inoculated with the starter culture at a volume ratio of 1:10. The standard fermentation process can be divided into three major stages: growth phase (90 min), E-lysis phase (120 min) and downstream processing.

The overall timeline for the production process is designed in such a way as the time from the automatic inoculation of the starter culture to the final concentration of the product the whole process takes 18 h and can be performed in one working cycle. The key events of the BG production process (Fig. 4) are discussed in more detail below.

Growth phase. The growth phase in an example fermentation with E. coli harboring plasmids for temperature-inducible E-lysis is conducted at 35°C, pH 7.20 and aeration parameters sufficient for exponential growth. To maintain a level of dissolved oxygen (dO₂) of approximately 20% saturation both stirring and aeration rate are adjusted gradually over the course of the growth phase. After 90 min E-lysis of E. coli is induced at cell densities of approximately 1–2 x 10⁹ cells/ml.

When recombinant proteins are expressed to become incorporated into the envelope complex (before E-mediated lysis) expression of the corresponding genes is induced chemically 30 min after inoculation (e.g., lac-, arabinose induction system). If synthesis of the foreign proteins slows down the growth rate so that lower cell densities are reached, this growth phase may be prolonged up to 120 min to compensate and increase the BG yield.

E-lysis phase. E-lysis of the culture is induced by temperature up-shift from 35 to 42°C (Fig. 3a). Currently, it takes roughly 10 min to reach the new temperature in the fermenter. During this time, stirring and aeration control is locked to prevent foaming during BG formation. The dO₂-level subsequently drops below 5% and so remains for about 30 min. In the fermentation log visual evidence for E-lysis onset is a sudden signature increase of dO₂ (Fig. 3b). The E-lysis phase continues for a total of 120 min with its end being characterized by the dO₂ reaching a stationary value of >95% saturation (Fig. 3c).

Down-stream processing. The BG product is harvested from the fermenter via tangential flow filtration (TFF) in a 0.2 µm hollow fiber module at a temperature of 15°C. Firstly, the fermentation broth (20 l) is concentrated to 2.0 l (Fig. 5a) and transferred
Process and quality control. During fermentation all relevant process parameters (T, pH, dO2, aeration, stirring) are monitored and controlled. Starting from the time-point of inoculation (Fig. 3A, -90 min) samples are taken every 30 min over the course of the fermentation (Fig. 3B–H) and analyzed for optical density (OD600) and colony forming units (cfu). All samples are also examined by light-microscopy and flow cytometry. Optionally, the biomass is also investigated for DNA content by real-time PCR and the level of protein E expression.

In standard fermentations with E. coli, the onset of E-lysis is linked to a sudden drop in OD600 of the culture broth and this simple determination is an important indicator of successful E-lysis induction. BG formation can also be observed as the appearance of translucent bacterial bodies in light microscopy. Both methods are good indicators for the quality of E-mediated lysis of E. coli but contain no quantitative information.

The TFF procedure as described above is an alternative to harvesting and washing BG via centrifugation. In contrast to the filtration process, this centrifugation is more laborious, time-consuming and might lead to BG aggregation because of difficulties with a proper re-suspension of the BG pellet. Another advantage of the implementation of TFF for harvesting and washing of BGs keeps all processes in a closed system and reduces the risk of cross-contamination during the handling procedure.

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actual E-lysis efficiency is determined by cfu counting one day after sample collection. Flow cytometry has been established as a reliable real-time tool for the assessment of E-lysis onset and the actual E-lysis efficiency is determined by cfu counting one day after sample collection. Flow cytometry has been established as a reliable real-time tool for the assessment of E-lysis onset and the progress of BG formation. For flow cytometry diluted samples are stained with two fluorescent dyes and run through a CyFlow analyzer (Partec, Münster, Germany). The first dye (RH414) stains phospholipid membranes and its fluorescence signal defines a gate for the exclusion of all non-cellular background. The discrimination of living cells, dead but non-lysed cells and BGs is achieved by a combination of the forward scatter signal (FSC) and the fluorescence signal (FL1) of the second dye [DiBAC(3)] which stains only cells that have lost their membrane potential. DiBAC-negative cells with a high scatter signal represent living cells, DiBAC-positive cells with a similar scatter signal represent the dead cell fraction. DiBAC-positive cells with a diminished scatter signal are identified as BGs. The scatter signal represents the dead cell fraction. DiBAC-positive cells with a similar membrane potential. DiBAC-negative cells with a high scatter signal represent living cells, DiBAC-positive cells with a similar scatter signal represent the dead cell fraction. The flow cytometry result for a given sample is available in less than 10 min after sampling. Representative dot-plots of an E. coli culture growth during the E-lysis process is shown in Figure 6 for the time-points induction (a), course (b) and end of the E-lysis phase (c).

After lyophilization the dry BG product is investigated with respect to sterility and re-susceptibility. For sterility investigations, 10 mg of BGs are re-suspended in rich medium and aliquoted for both nutrient agar plating and enrichment cultures. All sterility tests are performed in triplets to ensure that the final product does not contain any viable cells. The re-susceptibility is evaluated via flow cytometry with a lyophilized sample after re-suspension in dH2O. Since lyophilized BGs generally are easily rehydrated, the sample should give a similar picture and corresponding particle counts as the original sample.

**BG—Inactivation**

For the last 2 years, a new quality criterion stipulated that the harvested BG product should be free of any living cells before lyophilization. Although the efficiency of BG formation reaches three to five orders of magnitude during the time window of E-lysis (Figs. 3 and 4), any remaining live cells must be inactivated subsequently. The presence of protein E in the envelope complex of bacteria does not necessarily kill all bacteria by E-lysis. However, protein E in the membrane renders all bacteria more acutely sensitive to killing by lyophilization and in the past no living cell counts could be detected in the lyophilized BG samples. In applications where nucleic acid-free BGs are produced, inactivation can be accomplished by the expression of an additional “kill gene” in the host cells in combination with E-lysis. For this, the staphylococcal nuclease A (SNUC) is used, which reduces the DNA content below the detection limit of real-time PCR. SNUC activity is also responsible for cleaning up residual DNA in BGs and can lead to complete inactivation of the culture as it degrades the host DNA into fragments no longer than 100 base pairs. Activation of the positive effect of SNUC expression, minimizing both cell viability and residual DNA-content in the BG product, is dependent on the addition of Mg2+ and Ca2+ as well as a shift in pH to 8.0. The presence of BPL causes alterations (transition mutations, cross-linking, nicks) in nucleic acids. The presence of water fully hydrolyses BPL at room-temperature into non-toxic β-hydroxypropionic acid. Addition of the alkylating agent β-propiolactone (BPL) after harvesting is effective in fully inactivating all viable cells either in combination with or as an alternative to SNUC. BPL is known to react with nucleic acids, mainly guanine. BPL is widely used for the inactivation of viruses and further to sterilize vaccines, human tissue implants and plasma.

**Figure 6.** Flow cytometry pictures following the progress of lysis in an E. coli NM022 culture (pGLysivb); R1: living cells, R2: dead but intact cells, R3: lysed cells (BG); RN6: exclusion of non-cellular background with RH414 (not shown); FSC - forward scatter, FL1 - fluorescence intensity by DiBAC(3); (a) sample D (0 minutes, lysis induction), (b) sample E (30 minutes), (c) sample H (120 minutes, end of lysis phase).
The free DNA—has been removed from the product. Two equal doses of BPL given at 30 min intervals are sufficient for total inactivation of all surviving cells at 42°C within 60 min. The final BG product is washed with another 5.0 l dH₂O by dialfiltration before dispensing into aliquots for lyophilization.

**BGs—Applications**

**BGs solo.** Immunization against pathogenic Gram-negative bacteria using BGs has been studied in various animal models. BGs have been used in model investigations for human lung pathogens and for the development of veterinary vaccine candidates vaccination of swine with *A. pleuropneumoniae* (App). AppBGs resulted in protection against aerogenic infection with the potentially lethal pathogen. They also prevented colonization of the lungs and tonsils which indicated that immunization with BGs is superior to treatment with bacterins. More importantly, no clinical side-effects have been reported.

The application of BGs via mucosal inoculation is superior to parental inoculation. The mucosal application of AppBGs as oral immunization⁴⁴ or as aerosols⁴⁵ induced sterile immunity and cross-protection against other serotypes in pigs⁴⁶ whereas intramuscular immunization⁴⁶ fully prevented the vaccinated pigs against the disease after lethal challenge but did not confer sterile immunity because the challenge bacteria could be re-isolated from the tonsils from the vaccinated pigs.

BG produced from *P. multocida* and *M. haemolytica* (formerly: *P. haemolytica*) were used in rabbit and mice models. The antibodies produced were cross-protective; effective not only against the strain used for immunization but also against other Pasteurella strains.⁴⁷ *M. haemolytica* BG immunization of cattle offered protective immunity comparable to commercially available vaccines.⁴⁸ For *V. cholera* pre-clinical studies have been completed. The ilea loop challenge model revealed full protection of rabbits.

Interestingly, partial cross-protection between the classical O1 strain and the new upcoming O139 strain was observed. In most models mucosal application has proven to be a favorable route for administration of BG candidate vaccines inducing both humoral and cellular immune response.²⁹,³⁶

**BGs as adjuvants.** The BG morphology is not subject to denaturation during the lysis process. Thus all major immune stimulating elements are preserved. Those elements are referred to as pathogen-associated molecular patterns (PAMPs) and include lipopolysaccharides (LPS), monophosphoryl lipid A (MPL), peptidoglycan or flagella. As PAMPs are recognized by toll-like receptors (TLR) they trigger also the innate immune response. Consequently, all bacterial strains from which the BGs are derived induce innate immune reactions (Abtin, Koller, Lubitz, personal communication) as first response. They also carry intrinsic adjuvant properties which makes them extremely versatile to induce specific humoral and cellular immune responses in experimental animals.²⁹

**BGs as carriers of foreign protein antigens.** Using recombinant DNA technology, foreign antigens (AGs) can be incorporated into or become associated with the envelope complex of the
bacteria before lysis and become elements of the BGs (Fig. 2A). AGs may be presented on the cell surface via fusion with outer membrane proteins (e.g., ompA) or on the IM as membrane anchor fusions with N- or N/C-terminal targeting. Fusion with these membrane anchors did not affect proper folding and assembly or diminish the functionality of enzymes supporting the assumption that AGs are in their correct conformation. In addition to directly fusing the target AG to the membrane anchor, a system for subsequent loading of BGs with AGs was developed. In this approach the BGs are equipped with membrane-anchored streptavidin. After lyophilization such streptavidin BGs can be loaded with a desired biotinylated compound.

Another method of incorporating foreign proteins into BGs is the directed export to the PPS via MalE fusion proteins or PPS signal sequences. The PPS is sealed during lysis and the vast majority of all periplasmic components are retained within the envelope complex. The membrane-derived oligosaccharides of the PPS provide a protective environment against inactivation during lyophilization.

Fusions of target antigen DNA sequences with the bacterial surface layer (S-layer) genes sbsA or sbsB of Bacillus steaetherophilus, when expressed heterologous in Gram-negative bacteria, form sheet-like self-assembling superstructures within the cytoplasmic space. Since S-layers are made up of several 100,000 subunits, they are not expelled with the cytoplasm during E-lysis. Both S-layer genes accept insertion of foreign sequences coding for large foreign proteins. Linking MalE to SbsA the protein subunits can also be exported to the PPS prior to S-layer formation. All different options of AG presentation in BG envelopes are summarized in Figure 2A as a schematic drawing.

**BGs as carrier of biologically active substances.** The BG system provides a new promising platform for the delivery of drugs and other biologically active substances. BGs are devoid of any cytoplasmic content so the carrier capacity of the inner cytoplasmic lumen provides an intracellular space of approximately 250 femtoliter per BG. This lumen can be filled with drugs of interest as liquid or absorbed to the lipid compartment (independent of the inner volume) or specifically attached to receptors presented in the BGs.

BGs produced from Bacillus subtilis or Bacillus stearotherophilus, when expressed heterologous in Gram-negative bacteria, form sheet-like self-assembling superstructures within the cytoplasmic space. Since S-layers are made up of several 100,000 subunits, they are not expelled with the cytoplasm during E-lysis. Both S-layer genes accept insertion of foreign sequences coding for large foreign proteins. Linking MalE to SbsA the protein subunits can also be exported to the PPS prior to S-layer formation. All different options of AG presentation in BG envelopes are summarized in Figure 2A as a schematic drawing.

**BGs as carriers of DNA vaccines.** Conventional viral and bacterial vaccine delivery systems with high transfection efficiencies bear a risk of reversion to their original pathogenic forms. “Safer” non-viral systems such as attenuated bacteria, polycation/DNA complexes, nucleoporation have reduced transfection efficiencies. The BG system represents an alternative to current viral and bacterial methods in vaccine development with a new highly efficient gene delivery platform. One of the biggest advantages of the new DNA-carrier system is the safety of BGs. Recent in vitro investigations proved that BGs have no cytotoxic or genotoxic impact on different types of human cells after mutual co-incubation. This observation was independent of the BG species used (Koller and Lubitz, personal communication).

Recently, DNA vaccines were approved for use in veterinary practice. DNA vaccines still require intensive research and improvements before they are considered safe for use in human medicine. One reason for this slow pace in development and licensing approval of DNA vaccines is the requirement of high plasmid dosages and low immunogenicity, most commonly attributed to the absence of efficient delivery system. Many experiments have been carried out in order to deliver DNA vaccines using BGs as carriers, and a simple procedure for loading BGs with plasmid DNA has been standardized. Lysophosphatidic BGs are re-suspended in DNA solutions followed by a couple of washing steps to remove unbound plasmid DNA from inside the BGs. The amount of DNA loaded inside the BGs is directly related to the concentration of DNA solution used. This loading procedure is very efficient and up to 6,000 midsize plasmid copies per BG can be loaded.

One of the main advantages of BGs is that they are non-living. They retain all of the surface morphological, structural and antigenic components of their living counterparts. BGs also have an outstanding loading capacity. The inner space of BGs empty envelope can be loaded with a combination of peptides, drugs or foreign DNA which gives us an opportunity to design new types of polyvalent vaccines. We have shown that BGs loaded with plasmid DNA encoding green fluorescent protein (GFP) are efficiently internalized and phagocytosed by both professional antigen presenting cells (APCs) and tumor cells. BGs were able to deliver the heterologous genes to both professional antigen presenting cells (APCs) and tumor cells.
non-dividing cells (monocyte-derived dendritic cells) and dividing cells (macrophages and melanoma). Study results showed that up to 82% of cells expressing the plasmid encoded reporter gene delivered by BGs. Importantly, no cytotoxic impact was observed on target cells. Intradermal and intramuscular immunization of Balb/c mice with BGs loaded with pCMV encoding beta-galactosidase stimulated more efficient humoral and cellular AG-specific immune responses than naked DNA. Beta-galactosidase-specific immune response was detected after intravenous immunization of mice with autologous dendritic cells (DCs) transfected ex vivo with pCMV-beta-loaded BGs. An increase of IFN-γ/γ/γ compared to control was observed in animals vaccinated with DNA loaded BGs in response to restimulation by APCs pulsed with peptide containing the immunodominant MHC class I epitope. BGs enhanced expression of MHC class I molecules and costimulatory molecules on DCs. Cross-presentation of AGs delivered to DCs by BGs could activate both CD4+ and CD8+ T cells and stimulates the immune system to enhance immune response against AGs expressed by target cells. Bacterial LPS enhances maturation of DCs, affects endosomal acidification of DCs and also improves cross-presentation of AGs. Inner and outer membrane structures of BGs including LPS remain intact in BGs and the surface LPS effectively stimulate the AG-cross-presentation by DCs.

In general the production and loading of BGs with plasmid DNA are two separate tasks. With the introduction of our new self-immobilizing plasmids (pSIP) this multistep procedure was simplified into one step in vivo, cost effective procedure. During this process the plasmid DNA carrying an operator sequence is bound to a specific DNA binding protein present on the IM of the bacteria. The bacterial backbone sequences and antibiotic resistant genes are considered to be a biological safety risk for DNA vaccination and plasmid DNA used in gene therapy. To overcome this hurdle, new more sophisticated versions of pSIP BG-DNA-vaccines, based on minicircle (mc) DNA devoid of such biologically risky remnants were developed. This improved version of pSIP is based on the ParA resolvase system to produce mcDNA which is bound to the IM receptor. The corresponding sister pair miniplasmid produced during this process is expelled into the culture media during the gene-E mediated lysis. A modified system for minicircle production, digesting the miniplasmid has been reported, based on endonuclease activity of F-Scel gene encoded from parent plasmid. A new modified pSIP generation is currently under construction with encoded endonuclease activity to digest non-recombinant mother plasmids and the ParA produced miniplasmids.

The main benefit of DNA vaccines is the induction of both cellular and humoral immune responses. Processing of AG through both endogenous and exogenous pathways followed by AG epitopes present both MHC class I and class II molecules. Well designed and applied gene therapy should provide successful delivery of desired AG DNA to the APCs. This is followed by its expression, naturally processing and presentation of AG-derived epitopes. T cells raised against delivered, naturally processed and presented AGs by APCs might be more effective in recognition of the same epitopes presented by cells expressing identical AGs. The expression of a delivered gene should induce strong immune responses or change the behavior of targeted cells. BGs with their intact envelope structures include peptidoglycan and LPS. These elements are not only “waking up” professional phagocytic APCs but are also providing stimulatory impulses to tumor cells. It is known that e.g., melanoma cells have the capacity to behave as non-professional APCs and can phagocyte both apoptotic and live cells and as recently shown respond to challenge by BGs. Despite the high DNA loading capacity of BGs, relatively low concentrations of DNA are sufficient for effective gene delivery and expression by melanoma cells. High transfection efficiencies were obtained after incubation of BGs with melanoma cells. Similar results were seen with monocyte-derived DCs encouraging us to design BGs carrying selected immunogenic and immunodominant AGs. These DNA loaded BGs would be used simultaneously for gene transfer to both professional APCs and to tumor cells to induce or amplify AG-specific immune responses.

White biotechnology—BGs as micro-bioreactors for enzymatic reactions. Another possible application for the BG platform is the use of BGs as enzyme carriers. The lack of cytoplasm and of a membrane potential due to E-mediated lysis of the bacteria does not lead to a total loss of enzymatic activities. The enzymatic activities of BG membrane-bound β-galactosidase and chloramphenicol acetyl transferase have been described. Membrane associated enzymes like AT Pases are still functionally active in BGs. Moreover, even though the cytoplasmic content is expelled during lysis, the inside of the cytoplasmic membrane and its associated products are retained. As the IM and OM are fused at the border of the E-specific lysis tunnel enzymes from the PPS like alkaline phosphatase and β-lactamase are largely retained and active. Potential enzymes [e.g., ATPase and β-lactamase sustained relative activities in suspended BGs even after one week storage at 4°C. Enzyme activities were also detectable in lyophilized BG-batches stored long-term at ambient temperatures. The enzyme activities were similar to those of recently produced freeze-dried samples, e.g., ATPase activity no significant differences in enzyme activity were observed after five years of storage (Koller, Lubitz—personal communication). This data confirms that BG enzymes stay functionally preserved during long storage, which indicates the potential of BGs as reservoirs for biological functions e.g., for dietary enzyme substitution or for other use.

BGs can act as micro-reactors which follows the idea of Pründer et al. of producing enantioselective fine chemicals such as e.g., asymmetric synthesis of a 3,5-dicarboxyhydroxylate in biphasic ionic liquid/water systems. Potent enzymes [e.g., specialized alcohol dehydrogenases (ADHs)] are anchored to the IM while the internal lumen of the BG becomes the reaction space. Re-suspension of the BGs in an aqueous solution with a suitable reduction equivalent allows for proper function of the desired enzyme. Both product and educts in these kinds of reactions are often poorly water-soluble. For this reason, the use of a non-polar solvents such as a suitable ionic liquid (IL)—is essential. IL are organic salts which are liquid at ambient temperatures; due to their low vapor pressure they are considered...
as safe (“green solvents”). They feature good in-situ extraction properties for product recovery.92 BGs loaded with the reduction equivalent solution are dispersed in the ionic liquid. Thus this BG system provides the substrate and receives the product. It was shown that the enzyme activity of β-galactosidase could be vastly increased in an IL environment.93 Preliminary studies of our lab have demonstrated that β-galactosidase was active when BGs were re-suspended in the IL.94

Qualitative determinations showed successful hydrolysis reactions of the substrates which were delivered in the ionic liquid. These findings give first indication of the feasibility and attainable enzymatic activity of such approaches. When a multi-step enzyme system is introduced a limited series of reactions could be performed within one BG. Therefore the BG system could become a versatile vehicle in white biotechnology.

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References


1.2 Fermentation principles

Fermentation in this context is the large-scale high-yield implementation of a biotechnological process integrating knowledge from both biotechnology and process engineering. Microorganisms are utilized to generate a desired product from a nutrient solution. Depending on the process the product may either be the cells themselves (e.g. bacteria) or something they produce (metabolites, proteins etc.). In either case it is the objective of a fermentation process to maximize the product yield while minimizing production time and costs. For recombinant products it may be necessary to separate microbial growth and generation of the product in two consecutive steps. The actual fermentation process is followed by an array of downstream operations for product refinement and recovery.

Fermentation is performed in closed sterile vessels (fermenters). Different modes of operations are established for various applications. A common fermentation methods will be presented in this work: aerobic fermentation of bacteria in a stirred tank reactor (STR).

1.2.1 Stirred tank reactor basics

An STR is a tempered glass or steel vessel equipped with an agitator shaft for mixing of the liquid content. Aerobic operations also require a sparger to aerate the medium with a suitable gas or gas mix (air, pure oxygen etc.). STRs can be run in three different modes: single batch, fed-batch or continuous mode. The latter case is called continuous stirred tank reactor (CSTR).

A CSTR receives a stream of fresh sterile medium \( Q_{in} \) and delivers an equal stream of culture broth \( Q_{out} \) at the same time. In a well-run process (assuming ideal mixing of the liquid) the CSTR is at steady state. Steady state means that at all times and in all places the conditions inside the liquid phase are idem implying that the concentrations of all components in the culture broth do not change with time. The CSTR provides a comprehensive model from which the other operational modes (batch, fed-batch) can be derived. Figure 1.2 shows the rudimentary flow chart of a CSTR. Knowing that \( Q_{in} = Q_{out} = Q \) we can state that the volume is constant: \( V = \text{const} \). The mass concentration balance of any component \( A \) inside the control
volume (dotted line) can now be calculated from the following differential equation:

\[
\frac{d (AV)}{dt} = QA_{in} - QA + r_{pA} V - r_{cA} V = 0
\]  

(1.1)

\( r_{pA} \) and \( r_{cA} \) represent the rates of production and consumption of component \( A \), respectively. Expanding and re-arranging gives:

\[
\frac{dA}{dt} V + \frac{dV}{dt} A = Q (A_{in} - A) + (r_{pA} - r_{cA}) V = 0
\]  

(1.2)

### 1.2.2 Batch fermentation

In a batch fermentation all required nutrients are provided in the vessel. After inoculation the bacteria grow until the limiting substrate \( S \) is depleted. The initial concentrations for biomass and the limiting substrate at inoculation of the vessel are denote with the suffix 0 (\( X_0 \) and \( S_0 \)). During a batch fermentation no streams enter or leave the vessel and thus the volume does not change with time. Equation 1.2 is reduced to:

\[
\frac{dA}{dt} = (r_{pA} - r_{cA})
\]  

(1.3)

For calculation of the biomass concentration \( X \) in a batch fermentation we substitute \( r_{pA} = \mu X \) and \( r_{cA} = k_d X \). Neglecting bacterial death (\( k_d \ll \mu \)) equation 1.3 becomes:

\[
\frac{dX}{dt} = \mu X
\]  

(1.4)

Integration of this equation gives the relation for bacterial growth in batch fermentation.

\[
X = X_0 e^{(\mu t)}
\]  

(1.5)

For calculation of the limiting substrate concentration \( S \) in a batch fermentation we sub-

\[
\frac{dS}{dt} = q_s X
\]  

(1.6)

More commonly used is the calculation of the substrate concentration using the yield coef-

\[
\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \frac{dX}{dt}
\]  

(1.7)

Integrating gives:

\[
S - S_0 = -\frac{1}{Y_{X/S}} (X - X_0)
\]  

(1.8)
Using equation 1.5 and rearranging:

\[ S = S_0 - \frac{1}{Y_{X/S}} X_0 e^{(\mu t)} - 1 \]  

(1.9)

Since the product from the process discussed in this work is the cells themselves the mass balance for product formation is neglected.

### 1.2.3 Fed-batch fermentation

A batch process is limited with respect to productivity because the medium can only be loaded with nutrients to a certain extent. High substrate concentration may be inhibitory or even toxic. In order to reach high cell densities a fed-batch strategy can be chosen. The process starts with a regular batch phase as described in chapter 1.2.2. The fermenter is not filled up but rather to 30 ... 50% of the maximal working volume. After the limiting substrate is fully depleted a high-concentrated feed stream (large \( S_{in} \)) is activated to maintain bacterial growth. In order to keep the bacteria growing exponentially the feed rate also has to be increased exponentially. The chosen feed rate enforces the growth rate \( \mu_f < \mu_{max} \) on the system.

The initial conditions at the start of the feed stream are denoted with the suffix 1 (\( X_1, V_1 \) and \( S_1 \)). During the feed phase a feed stream \( Q_{in} = Q \) enters the vessel but no stream leaves it. Hence, the volume is no longer constant but changes with time. From equation 1.2 we get for the feed phase:

\[
\frac{dA}{dt} V + \frac{dV}{dt} A = Q A_{in} + (r_{pA} - r_{cA}) V
\]

(1.10)

Assuming a sterile feed stream (\( X_{in} = 0 \)), substituting \( r_{pA} = \mu_f X \) and again neglecting bacterial death the biomass balance can be written as

\[
\frac{dX}{dt} V + \frac{dV}{dt} X = \mu_f X V
\]

(1.11)

Integrating this equation gives the relation for bacterial growth during the feed phase:

\[
\frac{X}{X_1} = \frac{V_1}{V} e^{(\mu_f t)}
\]

(1.12)

Since the growth rate during the feed phase is \( \mu_f < \mu_{max} \) the limiting substrate cannot accumulate and thus \( S = 0 \) at all times. This means that the left hand side of equation 1.10 becomes zero for the balance of limiting substrate. With \( r_{pA} = 0 \) and \( r_{cA} = -q_s X \) we get:

\[
0 = Q S_{in} + q_s X V
\]

(1.13)
Equating equation 1.6 with 1.7 we get:

\[ q_s X = - \frac{1}{Y_{X/S}} \frac{dX}{dt} \]  

(1.14)

Combining this relation with equation 1.4 equation 1.13 becomes:

\[ 0 = QS_{in} - \frac{1}{Y_{X/S}} \mu_f XV \]  

(1.15)

Re-arranging for the feed stream:

\[ Q = \frac{\mu_f XV}{Y_{X/S} S_{in}} \]  

(1.16)

With \( X_1 \) and \( V_1 \) the initial feed stream \( Q_1 \) can be calculated. Using the initial conditions and the biomass balance (equation 1.12) we get the relation for the feed stream:

\[ Q = Q_1 e^{(\mu_f t)} \]  

(1.17)

With the information from equations 1.5, 1.9, 1.12, 1.16 and 1.17 the behavior of a fed-batch system can be modeled. Figure 1.3 shows a virtual fed-batch system for bacterial growth on glucose. The initial parameters chosen for the model are: \( V_0 = 8.0 \) l, \( S_n = 20.0 \) g/l, \( X_n = 0.1 \) g/l and \( \mu_{\text{max}} = 0.80 \) h\(^{-1} \). With a yield coefficient of \( Y_{X/S} = 0.45 \) the biomass...
concentration at the end of the batch phase is $X_i = 9.06 \text{ g/l}$. The feed conditions are set to $S_{in} = 200.0 \text{ g/l}$ and $\mu_r = 0.40 \text{ h}^{-1}$. Given a final fermentation volume of $V = 20.0 \text{ l}$ the biomass concentration can be increased to $X = 57.89 \text{ g/l}$.

1.3 Flow cytometry

Flow cytometry (FCM) is a method for measuring properties of individual cells in a suspension. A flow cytometer consists of the fluidics system passing the sample through the instrument and the optical bench where signals are generated and recorded. The core of the fluidics system is the cuvette. The sample stream is injected to the bottom of the cuvette where it is hydrodynamically focused through the drag created by the so-called sheath fluid. Assuming laminar flow in the cuvette the sample stream and the sheath fluid do not mix which ideally results in a stream of single particles traveling up the cuvette [42]. The optical bench consists of a laser and an array of lenses, filters and photomultiplier tubes (PMTs). The laser is aligned and focused so that it crosses the sample stream with an elliptical focal spot. Scattering as caused by cells passing the laser beam is collected in line with the laser beam (forward scatter, FSC) and perpendicular to it (side scatter, SSC). Using dichroic filters in the perpendicular light path fluorescence signals (FL) at different wavelengths can be collected as well [45]. Fluorescence of the cells is either intrinsic (e.g. GFP) or due to preceded staining with suitable fluorescent dyes [24].

![Figure 1.4: Schematic setup of a flow cytometer](image_url)
Figure 1.4 shows the schematic setup of a flow cytometer (not drawn to scale) with FSC, SSC and three dichroic filters for fluorescence detection (FL1, FL2 and FL3). In biotechnological applications flow cytometry integrates analysis of cell counts with cell viability or other properties. Depending on the assessed parameters one sample run may replace multiple testings. Assays for cell viability [9, 22, 23], DNA and protein content [6] and others have been described. Depending on the availability of a suitable dye many cellular properties are detectable [24].

FCM is anticipated to be superior to conventional culture techniques in such cases where cells may not be able to sustain growth on solid media or where time-dependence is an aspect [9]. FCM evaluates large populations (several hundred thousands of individual cells per run) making the results statistically reliable.

1.4 Radio-frequency impedance measurements

On-line determination of viable biomass concentration can be done through capacitance measurement, also referred to as radio-frequency impedance (RFI) measurement. Intact cell membranes have insulating properties and therefore the ability to separate charges. When an intact cell is exposed to a radio frequency field such charge separation is affected across the cell membrane. This leaves the cell an induced dipole that can be compared to a small capacitor [8]. The virtually immeasurable capacitance of single cells adds up so the more viable cells are present in the medium the higher the overall capacitance.

Figure 1.5 shows the measuring principle adapted from Kaiser et al. Cells with a disrupted or ‘leaky’ membrane (depicted in gray) do not contribute to the overall capacitance and are virtually invisible to this method [25]. This makes the RFI method of special interest for BG fermentations since the cells loose their membrane potential during the process and therefore the capacitance should drop with the rate of E-lysis.

The dielectric properties of a cell suspension are dependent on the frequency of the electric field. Lower frequencies charge up the cell membrane capacitance more so that plotting the measured permittivity over the log
frequency in a range of 0.1 to 20 MHz gives an inverted sigmoid curve with a low plateau for higher frequencies and a high plateau for lower frequencies [1]. This effect is called β-dispersion; the inflection point of the sigmoid curve is called the critical frequency \( f_c \) (see fig. A.1, appendix). The dielectric increment between the two plateaus is defined by the following equation [8]:

\[
\Delta \varepsilon = \frac{9}{8} P \frac{d_p C_m}{\varepsilon_0}
\]

\( P \) is the volume fraction of cells in the suspension (dimensionless) while \( d_p \) is the cell diameter, \( C_m \) the specific membrane capacitance per unit area and \( \varepsilon_0 \) the permittivity of free space. The latter are all constants at a given setup meaning that magnitude of the dielectric increment is directly proportional to \( P \). Calibration allows to convert into the measured capacitance (pF/cm) displayed by the measuring amplifier [1]. Measurements should be conducted at frequencies < \( f_c \), for \( E. coli \) measurements at 1.0 MHz are suggested [1].

### 1.5 Tangential flow filtration

Tangential flow filtration (TFF) is a filtration method where - as opposed to dead-end filtration - the direction of the feed stream is tangential and not perpendicular to the filter surface. This method offers great advantage in some applications. The feed stream can be circulated an thereby concentrated. The feed stream also carries off particles that are not able to penetrate the membrane so that fouling (build-up of a filter cake) is only a minor issue. Consequently, the pressure loss across the membrane remains broadly constant. The liquid stream crossing the membrane is called permeate while the retained feed suspension is called retentate. TFF systems utilize different membrane modules; most prevalent are membrane cassettes and hollow fiber modules. Figure 1.6 shows a hollow fiber module and a cross sectional view into one individual hollow fiber.

The driving force pushing the permeate through the membrane is the trans-membrane pressure (TMP). TMP is defined as the pressure difference between both sides of the membrane. Since TMP cannot be measure easily it is usually estimated from the pressures at the inlet (\( p_i \)) and outlet (\( p_o \)) of the membrane module and the permeate pressure (\( p_o \)).

\[
\text{TMP} = \frac{1}{2} (p_i + p_o) - p_o \tag{1.18}
\]

The pressure loss inside the TFF module is simply:

\[
\Delta p = p_i - p_o \tag{1.19}
\]
As the retentate stream is recirculated through the TFF module the total liquid volume is reduced and the retained particles are concentrated. This operation mode is called concentration. In another application the liquid volume that is withdrawn as permeate is replenished with unloaded liquid (water, buffer) at an equal rate in order to keep the feed volume constant. The feed is washed or re-buffered, respectively; this operation mode is referred to as dia-filtration (DF).

1.6 β-propiolactone

The organic compound β-propiolactone (BPL) has been described as a chemical inactivant. Its chemical structure features a four-membered ring that is shown in figure 1.7. BPL has alkylating properties and is highly reactive with nucleophilic components such as nucleic acids. It is known to attack and alter the DNA; the main target for BPL action at low concentrations are purine residues (i.e. guanine). BPL-caused alkylations result in GC-AT transition mutations as well as inter- and intrastrand looping of the double helix [39]. This leaves the DNA non-reproducible and eventually result in cell death.

Due to this properties BPL is 'reasonably anticipated to be a human carcinogen' by the authorities [37]. In aqueous environments BPL is gradually hydrolyzed forming non-hazardous 3-hydroxypropionic acid (3-HPA, see figure 1.7) without further additives required. Both the rate of DNA-inactivation and the hydrolysis are temperature-dependent and advance faster at higher temperatures. At 37°C complete degradation of BPL takes about 3 h [39].
As mentioned before BPL is used as a chemical inactivant, e.g. for virus inactivation in blood plasma or vaccines [7, 26, 29, 40].

1.7 References


2 Materials and methods

2.1 Fermenter systems

2.1.1 Labfors 3

Preliminary small scale experiments are performed in a 7.5 l tabletop fermenter Labfors 3 (INFORS-HT, Bottmingen, Switzerland). The fermenter is used with a working volume of 4.0 l to give a scale ratio of 1:5 with the Techfors-S fermenter (see section 2.1.2). With the inner diameter of the fermenter $D = 150$ mm and the fermenter geometry the filling height is $H = 325$ mm. The agitator shaft is equipped with two Rushton turbines ($d = 55$ mm) mounted at 275 and 390 mm from the lid.

The fermenter has regular temperature-, pH-, stirring- and aeration-control that can either be controlled directly at the fermenter control panel or through the process control software IRIS V.5 (INFORS-HT). Temperature is controlled with tempered water through the glass double jacket. The fermenter has a built-in Pt-100 temperature probe, pH-control is actuated by two built-in digital peristaltic pumps for acid and base addition.

**Equipment: Labfors 3**

The pH-probe is a single-rod measuring cell 405-DPAS-SC-K8S/425 (METTLER TOLEDO, Greifensee, Switzerland). The oxygen-probe is an optical sensor VisiFerm DO 425 mm (HAMILTON, Bonaduz, Switzerland) equipped with a VisiFerm Power Adapter (HAMILTON) for compatibility with the D4/T82 connector at the Labfors. The air flow is controlled by an red-y GSC-C3SA-BB26 mass flow controller (MFC; VÖGTLIN, Aesch, Switzerland).

2.1.2 Techfors-S

Pilot scale experiments are performed in a 30.0 l stainless steel fermenter Techfors-S (INFORS-HT). The fermenter is operated with a maximal working volume of 20.0 l. The vessel has an inner diameter of $D = 250$ mm. At the maximum liquid level of 20.0 l the filling height
is \( H = 450 \) mm. The Techfors-S fermenter has a built-in Pt-100 temperature probe and two digital peristaltic pumps for pH-control (acid/base). Temperature control is done with tempered water and/or steam through the steel double jacket.

Experiments are conducted in two separate Techfors-S fermenters, subsequently called Techfors 01 (TF1) and Techfors 02 (TF2). The fermenters are identical in their geometry. Deviating equipment for both fermenters will be discussed in the following.

**Equipment: Techfors 01**

In standard batch fermentation the agitator shaft is equipped with two Rushton turbines (\( d = 80 \) mm) mounted at 300 and 550 mm from the lid. TF1 has a steamable ID 17 mm harvesting outlet (2” tri-clamp flange) at the bottom of the vessel as well as an ID 17 mm recirculation port (2.5” tri-clamp flange) on the top left hand side of the vessel. The sampling port in this case is installed in one of the four 50 mm thread ports at the lower front of the vessel.

The pH-probe is a single-rod measuring cell 405-DPAS-SC-K8S/120 (Mettler Toledo). The oxygen-probe is a InPro 6800 120 mm Clarke electrode (Mettler Toledo). The probes are placed in 50 mm thread ports as well. Aeration of the fermenter is solely done with compressed air. The air flow is controlled with a F-201AV-FFV-33-V MFC (Bronkhorst, Ruurlo, The Netherlands). All control actions can be done directly at the fermenter control panel or through the process control software IRIS V.5

**Equipment: Techfors 02**

For fed-batch fermentation a third Rushton turbine is mandatory. In this case the stirrers are mounted at 370, 470 and 570 mm from the lid.

TF2 does not have a harvesting port while the sampling port is located at the bottom of the vessel. A total of seven 50 mm thread ports is available at the lower front and rear of the vessel. The pH-probe is a single-rod measuring cell 405-DPAS-SC-K8S/120 (Mettler Toledo). The oxygen-probe is a Visiferm DO 120 mm (Hamilton) equipped with a Visiferm Power Adapter (Hamilton) for connection to the Techfors-S. The remaining 50 mm ports can be used to harbor various probes for on-line monitoring of the process (e.g. RFI measurements, see section 2.4).

The fermenter is aerated with compressed air and optionally with pure oxygen; both flows are controlled by red-y MFC’s (Vögtlin, air: GSC-C9SA-BB12, oxygen: GSC-C3SA-BB26). The head pressure in the fermenter is monitored and adjusted with a built in
control unit (pressure sensor/exhaust gas valve). All control actions can be done directly at the fermenter control panel or through the central process management system Lucullus V.3.1.0 (Biospectra, Schlieren, Switzerland).

Steam generator

Both Techfors-S fermenters are sterilized in-situ with steam. The steam is provided from a 10 kW steam generator (Infors-HT). The setup of TF1 utilizes steam for temperature control which allows for rapid T-shifts during fermentation. TF2 is designed to use tempered water for temperature control. Manually opening the steam admission valve rapid T-shifts can be implemented at TF2 as well.

2.2 Culture media

The recipes for all fermentation media, feed and stock solutions used in this work are given in the following sections. All media are supplemented with gentamicin stock (10 g/l gentamicin sulfate\(^a\)) to a final concentration of 20 mg/l after sterilization.

For pre-cultures the pH of the media is adjusted to pH 7.20 prior to sterilization. For the complex media 5M NaOH is used, for the DeLisa medium 5M KOH is used. The fermentation media are adjusted to pH 7.20 in the fermenter after sterilization by the pH controller using corrective agents.

All media, supplements and stock solutions are prepared with deionized water (d\(H\)_2O). Foaming is counteracted using different anti-foam agents. For the complex media traces of Antifoam A\(^a\) are added to the medium prior to sterilization, additional Antifoam A can be added upon demand. For the minimal medium sufficient amounts of poly-propylene glycol\(^b\) (PPG) are added to the medium prior to sterilization.

2.2.1 Complex culture medium: Luria-Bertani

The primary complex culture medium is low salt Luria-Bertani (LB) used in different variations. One variant is defined by exchanging tryptone with soy peptone. This ‘vegetarian’ version (LBv) is used as animal protein-free medium. For fermentations of \textit{Shigella flexneri} 2a featuring staphylococcal nuclease A the NaCl concentration is increased to 1% (LB\(^+\)). The recipes for all three variations of LB are shown in table 2.1.

\(^a\)ROTH, Karlsruhe, Germany
\(^b\)Sigma-Aldrich, Buchs, Switzerland
2.2.2 Complex culture medium: 'Ed Oaks'

Another complex culture medium is optimized for the growth of *Shigella flexneri* 2a. The recipe as shown in table 2.2 was developed by Dr. Edwin V. Oaks (Walter Reed Army Institute of Research, Silver Springs MD, USA) and subsequently named 'Ed Oaks' medium (EO). For fermentation the buffering components are excluded (EO\(^-\)). Pre-cultures are grown in two-fold concentrated medium (EO\(^+\)(2x)). The recipe for the different variations of EO medium are shown in table 2.2.

<table>
<thead>
<tr>
<th>Component</th>
<th>final conc. (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LB</td>
</tr>
<tr>
<td>Yeast extract (^a)</td>
<td>5.00</td>
</tr>
<tr>
<td>Tryptone (^a)</td>
<td>10.00</td>
</tr>
<tr>
<td>Soy peptone (^a)</td>
<td>-</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.00</td>
</tr>
</tbody>
</table>

Table 2.1: *Medium recipe for variations of the LB medium*

2.2.3 Minimal culture medium 'DeLisa'

High-density fermentation of *Escherichia coli* Nissle 1917 is performed in minimal medium. The recipe is adapted from DeLisa et al. [2] and subsequently named 'DeLisa' medium. The medium components are produced and sterilized separately and mixed for fermentation (described in detail below). The overall composition of the batch medium (20 g/l glucose) and feed solution (150 g/l glucose) is shown in table 2.3. For pre-cultures in DeLisa medium
the batch recipe is used with a reduced glucose concentration of 8 g/l.

Due to precipitation issues and premature reactions during autoclaving the DeLisa medium cannot be prepared in one step.

**Stock solutions**

Since the ratio of the trace element (TE) concentrations is in some cases different in the batch medium and the feed solutions some trace elements have to be prepared as single component stocks. The 'TE-stock' is a multiple component stock with all components that share the same ratio in batch and feed. Table 2.4 shows the different stock solutions and suggested sterilization method.

**Batch medium**

The medium for batch fermentations (ex.: 20.0 l batch) is prepared in two splits. The glucose is weighed in, dissolved in a split volume of 10% of the final fermentation volume (ex.: 2.0 l) and autoclaved separately. The basic medium components (i.e. phosphates and citric acid) are weighed in and dissolved in a split volume of 86.7% of the final fermentation volume (ex.: 17.34 l). The basic medium is transferred to the fermenter and sterilized in-situ. After sterilization the glucose solution is added to the basic medium through a sterile connection. In case the volume of the inoculum is not neglectable the split volume of the basic medium has to be reduced accordingly (ex.: 1.5 l inoculum - reduce volume of basic medium to 15.84 l).

The remaining 3.3% of the final fermentation volume (ex.: 0.66 l) are taken by the stock solutions. The respective amounts of the stocks as determined by the concentration factor and the final fermentation volume are mixed under the laminar flow and added to the medium through a sterile connection (ex.: EDTA, final conc. 0.0084 g/l, stock conc. 0.84 g/l, for 20.0 l: add 200 ml stock).

**2.3 Sampling and analysis**

Samples taken from the process are by default analyzed with respect to biomass, cell counts, cell viability and evaluated for appearance (not quantitative) under the microscope. Where necessary, the concentrations of glucose and acetate are monitored as well as the concentration of DNA in both supernatant and product.
## Process Development for Industrial Scale BG Production

Table 2.3: *Medium recipe for the DeLisa medium*

<table>
<thead>
<tr>
<th>Component</th>
<th>final conc. (g/l)</th>
<th>ster. method</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO$_4$ · 7 H$_2$O (stock 500x)</td>
<td>600.00</td>
<td>autocl. sep.</td>
</tr>
<tr>
<td>C$_6$H$_5$O$_7$ · Fe (stock 100x)</td>
<td>10.00</td>
<td>autocl. sep.</td>
</tr>
<tr>
<td>EDTA (stock 100x)</td>
<td>0.84</td>
<td>autocl. sep.</td>
</tr>
<tr>
<td>Zn(CH$_3$COO)$_2$ · 2 H$_2$O (stock 100x)</td>
<td>2.60</td>
<td>filter ster.</td>
</tr>
<tr>
<td>thiamine HCl (Sigma)</td>
<td>0.0045</td>
<td>-</td>
</tr>
<tr>
<td>poly-propylene glycol (AF, Sigma)</td>
<td>1.00</td>
<td>5.00</td>
</tr>
</tbody>
</table>

Table 2.4: *Recipes and sterilization methods for stock solutions (DeLisa medium)*

<table>
<thead>
<tr>
<th>Component</th>
<th>final conc. (g/l)</th>
<th>ster. method</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoCl$_2$ · 6 H$_2$O</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>MnCl$_2$ · 4 H$_2$O</td>
<td>3.00</td>
<td></td>
</tr>
<tr>
<td>CuCl$_2$ · 2 H$_2$O</td>
<td>0.24</td>
<td>filter ster.</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>Na$_2$MoO$_4$ · 2 H$_2$O</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>
2.3.1 Biomass and cell counts

The biomass concentration is determined from the optical density at $\lambda = 600$ nm (OD$_{600}$). The extinction of the culture broth is measured in a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). For extinction values > 1 the culture broth has to be diluted. For EcN the OD$_{600}$ has been correlated experimentally with the dry biomass (DBM, see appendix A.5.3). For DBM determination empty glass tubes are put to 95°C (drying oven) 24 h prior to the experiments. Before the measurements, the tubes are let cool down to room temperature and weighed empty. Then 5 ml of undiluted culture broth are transferred into the tubes and centrifuged at 5,000 rpm for 10 min. The supernatant is discarded and the pellet re-suspended in 5 ml fresh water. This washing step is repeated three times in total. After the last washing step the tubes with the wet pellets are put to 95°C for another 24 h. After letting them cool down to room temperature the tubes are weighed; the weight difference to the empty weight can be used to calculate the DBM as g/l.

Cell count analysis is performed as colony forming unit (cfu) determination from appropriate dilution of the culture broth. Dilution series are done in a combination of $10^{-1}$ and $10^{-2}$ steps in 0.85% (w/v) saline. 50 ml of the diluted broth are applied to Plate-Count-Agar plates with a WASP spiral plater (Don Whitley Scientific, Shipley, UK). Each sample is plated in quadruplicate. Counting is done with a ProtoCOL SR 92000 colony counter (Synoptics Ltd, Cambridge, UK). Re-calculating the cell counts with the dilution and sample volume gives the final cfu as cells per ml.

Cell counts and viability are also determined from flow cytometry (see section 2.3.2).

2.3.2 Flow cytometry

For flow cytometry a CyFlow SL system (Partec, Münster, Germany) is used. The machine is equipped with a 488 nm blue solid state laser and features true volumetric absolute counting (sample size: 200 µl). A total of five optical parameters is available including three colors. These parameters are forward and side scatter (FSC/SSC) as well as fluorescence channels 1 to 3 (FL1/FL2/FL3). The fluorescence channels detect the following wavelengths: FL1 - 527 nm (bandpass filter, bandwidth 30 nm), FL2 - 590 nm (bandpass filter, bandwidth 50 nm) and FL3 - 630 nm (longpass filter). For the assessment of BG formation parameters FSC, FL1 and FL2 are used.

Dilution of any sample has to be chosen so that the particle count rate can be held in a range of 800 to 1,100 per sec varying the sample speed between 0.5 and 3.0. Diluted
samples are stained using two fluorescent dyes (both: AnaSpec, Fremont CA, USA). The dye RH 414 (red, abs./em.: 532/718 nm) unspecifically stains plasma membranes. The dye DiBAC₄(3) (green, abs./em.: 493/516 nm) only penetrates and stains cells with a disrupted membrane potential. Staining with RH414 stock (2 mM) and DiBAC₄(3) stock (0.5 mM) is done at 0.75 to 1.50 µl/ml depending on the dilution. Neither dye requires incubation.

The FL2 channel (orange) is used to pick up RH414 in order to separate cells from non-cellular noise. A distinct peak in the FL2-histogram defines a gate for positive identification of cellular particles. This gate (RN6) is applied as a filter to all further analysis. The FSC signal for whole cells (‘full’) is generally higher than for BGs (‘empty’); even though cell size plays a certain role in FSC. The FL1 signal (green) allows for discrimination of viable and dead cells as DiBAC₄(3) stains dead cells only. Combining the information obtained from channels FSC and FL1 results in three possible combinations:

- R1 - high FSC signal (‘full’) and low FL1 signal (viable): whole live cell
- R2 - high FSC signal (‘full’) and high FL1 signal (dead): whole dead cell
- R3 - low FSC signal (‘empty’) and high FL1 signal (dead): Bacterial Ghost

In a dotplot of FSC (abscissa) vs. FL1 (ordinate) these property combinations define certain polygonal regions (R1/R2/R3) representing the populations of live cells, whole dead cells and BGs. During the process of BG formation the vast majority of cellular particles shifts from R1 to R3.

### 2.3.3 Detection of nutrients and metabolites

**Infinite 200 Microplate Reader**

The Infinite 200 Microplate Reader (Tecan, Männedorf, Switzerland) is a multimode reader featuring photometric, UV- and fluorescence measurements. Using the GlucPAP assay GL3981 (Roche Diagnostics, Basel, Switzerland) glucose concentrations in a range of 0.05 - 6.00 (g/l) can be determined photometrically. Higher concentrated samples are measured in 10⁻¹ dilutions. 5 µl of the (diluted) sample are pipetted into a flat bottom 96-well plate which is then brought to the sampling drawer of the microplate reader. Immediately before the measurement 180 µl of the enzyme reagent are pipetted into the well. The sample is incubated at 37°C for 10 min (shaking: 300 rpm). The extinction of the sample at λ = 505 nm is determined at end of the incubation phase and is directly proportional to the glucose concentration. The actual glucose concentration is calculated from a standard curve.
**CuBiAn XC Photometric Biochemistry Analyzer**

The CuBiAn XC Photometric Biochemistry Analyzer (Roche Diagnostics, Basel, Switzerland) is used for the automated detection of glucose and acetate concentrations in the culture broth during fed-batch fermentation. 1 ml of culture broth are centrifuged and the supernatant is taken for analysis. Samples may have to be diluted according to the measurement range of the test method and the expected concentration of the target substance. For glucose measurements two different methods can be chosen: a high sensitivity method (0.05 - 6.00 g/l) or a low sensitivity method (0.25 - 30 g/l). Both methods use the GlucPAP assay GL3981 (Roche Diagnostics, see also section 2.3.3). Measurements of the acetate concentration (0.05 - 6.00 g/l) are done using the Enzytec fluid Acetic Acid assay E5226 (r-biopharm, Damstadt, Germany).

In all cases the extinctions of the samples are compared with internal standard curves and the results are displayed as g/l.

**2.3.4 DNA measurements**

**DNA in the supernatant**

DNA concentration in the supernatant of the culture broth is determined photometrically. Extinction measurements are performed at $\lambda = 260/280$ nm in the Infinite 200 Microplate Reader (Tecan, see also section 2.3.3). 1 ml of (diluted) supernatant is pipetted to a UV-transparent well plate. With the given geometry of the wells the pathlength can be determined: $l = 0.622$ mm. The DNA concentration is calculated ($\mu$g/ml) from:

$$c_{DNA} = E_{260} \cdot 50 \cdot 0.622$$

Where necessary the results need to be corrected for the applied dilution. For reliable results the ratio of $E_{260} : E_{280}$ should be $> 1.8$

**DNA in the product: real-time PCR**

The DNA contents of BG products is determined through real-time PCR with an iCycler iQ™ RT-PCR Detection System and iQ™ SYBR green Supermix (Bio-Rad, Hercules CA, USA). Primers (stocks: 10 pmol/µl) specific for the gentamicin resistance cassette are GfwRT (5’-AGCGCGATGAATGTCTTACTACG-3’) and GrevRT (5’-TCCATGCGGGCTGCTTTAG-3’) yielding a 122 bp product. Standards are prepared from known plasmid
concentrations as $10^{-1}$ to $10^{-8}$ dilution series; standard curves with a correlation of $R^2 < 0.998$ are rejected.

The RT-PCR Mastermix is: 12.5 µl iQ™ SYBR green Supermix, 1 µl fw/rev primer (stock), each, 5.5 µl H₂O. For measurements 5 µl sample are mixed with 20 µl Mastermix. The RT-PCR protocol is - 1.: 95°C for 190 sec; 1x - 2.: 94°C for 40 sec, 60°C for 60 sec; 30x - 3.: 72°C for 300 sec; 1x - 4.: 95°C for 30 sec; 1x - 5.: 60°C for 30 sec; 1x, 6.: 60°C for 10 sec; 75x. Fragments are identified by melting curve analysis.

### 2.4 Radio-frequency impedance measurements

Viable biomass in the culture broth during fed-batch fermentation is determined on-line through capacitance measurements (radio-frequency impedance, RFI) using a Biomass monitor 220 (Aber Instruments Ltd., Aberystwyth, UK) with an annular probe. According to the manufacturers suggestion measurements with *E. coli* are performed at 1 MHz [1]. The signal is recorded together with the fermentation protocol through the Lucullus process management system. A correlation for DBM and RFI data (pF/cm) is shown in figure A.10 (a), appendix.

### 2.5 Harvesting of BG products

#### 2.5.1 Tangential flow filtration

A tangential flow filtration (TFF) system is used for dia-filtration (DF) and concentration of the culture broth. TFF is done in a microfiltration hollow fiber module CFP-2-E-8A (GE Healthcare, Chalfont St. Giles, UK) with a cut-off size of 0.2 µm. The filtration area is 0.36 m², the individual hollow fibers in the module are identical in geometry with a nominal flow path length of 30 mm and a fiber diameter of 1.0 mm. Two peristaltic pumps are available for the TFF system: a Flowmaster FMT 300 with a flow rate of up to 13.9 l/min and a MCP Standard with a flow rate range of 0.45 to 3,400 ml/min (both: Ismatec, Glattbruck, Switzerland).

For feed circulation through the TFF module the Flowmaster pump is used with ID 16 mm silicone tubes and PharMed Ismaprene SC0696 pump tubes (ID = 15.9 mm). The volumetric flow rate of the Flowmaster pump is implemented indirectly through the rotational speed of the pump head. Therefore a correlation curve linking the rotational speed and the volumetric
flow rate was recorded. The curve and the resulting calibration function are shown in appendix A.2.1.

Since the TMP cannot be accessed directly (as mentioned in chapter 1.5) the pressure difference $\Delta p$ between inlet and outlet of the module is used to define the setup of TFF. $\Delta p$ is measured with a digital manometer GDH 13 AN (Greisinger, Regenstauf, Germany). The pressure loss in the module can be manipulated by reducing and re-expanding the effective cross sectional area of the outlet tube downstream of the module. This is done by tightening/loosening a hose clamp around the outlet tube. The suggested values for $\Delta p$ for the different procedural steps represent experience values.

The MCP Standard pump is used for replenishing the liquid volume during DF with ID 3.0 mm silicon tubes and PharMed Ismaprene MF0012 pump tubes (ID 3.2 mm). For this pump calibration of the volumetric flow rate can be done via the device interface. Hence, any desired pump rate can be implemented directly as ml/min over the control display. DF aims to maintain a constant liquid volume in the feed container. The steady state between permeate and fresh water flux is monitored and manipulated manually. Therefore, every 7.5 min the permeate flux is collected in a measuring cylinder for 30 sec. The resulting nominal volumetric flow rate for the fresh water flux is then adjusted at the MCP Standard pump display.

All silicone tubes mentioned in this section are Rotilabo tubes (ROTH), all pump tubes are products purchased from Ismatec. Pictures showing the setup of DF applied during fermentation in a 20.0 l Techfors-S fermenter as well as for washing of 2.0 l BG concentrate are displayed in the appendix (fig. A.4 and A.5). Initial experiments for feasibility and selection of an appropriate membrane and cut-off size were done using an ÄKTAcrossflow™ automated filtration system (see fig. A.3, appendix).

### 2.5.2 Separator

For harvesting of the culture broth via mechanical separation a disk stack centrifuge CTC1-06-107 (GEA Westfalia Separator, Oelde, Germany) is available. The product broth is withdrawn from the fermenter through the harvesting outlet (driven by the head pressure in the vessel). The flow in and out the rotor of the separator are adjusted through membrane valves so that the supernatant is clear ($OD_{600} < 0.1$).

The collected product cannot be discharged under sterile conditions so the whole fermenter has to be harvested in one step. At the end of the procedure the rotor is removed from the separator and the product is collected under laminar flow using cell scrapers for tissue
2.6 References


3 Production of DNA-free *Shigella flexneri* 2a Bacterial Ghosts

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*Shigella flexneri* serotype 2a (Sf2a) is one major cause for shigellosis especially in developing countries. Based on the Bacterial Ghost (BG) platform system a candidate vaccine against Sf2a is suggested. In order to develop a safe vaccine a stable and reproducible production process for Sf2a (pGLNlc) BGs is presented. The targeted combination of three approaches, i.e. E-lysis, staphylococcal nuclease A (SNUC) activity and product inactivation with β-propiolactone (BPL), was used for BG production. After process development a consistency study was performed to demonstrate stability of the production process. The average yield of the process was \(8 \cdot 10^8\) cells per ml. The product consisted of \(99.94\% \pm 0.02\) BGs with all their anticipated beneficial properties such as preserved antigenic structures. SNUC activity reduced the DNA contents in the BG product by almost four and a half logs over the course of the production process. As the DNA concentration before harvesting was at the detection limit of real-time PCR the BG product was essentially DNA free. Final inactivation with \(0.75\%\) (v/v) BPL for 60 min resulted in a bioburden-free Sf2a (pGLNlc) BG product before lyophilization. Sterility of the lyophilized product was confirmed as well.
List of abbreviations


3.1 Introduction

Bacterial Ghosts (BGs) are produced from Gram-negative bacteria by a process called E-lysis. E-lysis originates from the expression of the cloned bacteriophage ϕX174 gene E product (protein E) [2,10]. Other than many bacteriophage lysis proteins protein E has no enzymatic activity [6,19]. It is a membrane protein that induces the fusion of the inner and outer membrane in Gram-negative bacteria [3]. Membrane fusion is limited to areas of potential cell division and results in a typical trans-membrane tunnel structure [30,31]. The main driving force for E-lysis is a difference in osmotic pressure between the cytoplasm and the culture medium. E-lysis is an explosive event rapidly releasing all cytoplasmic components to the surrounding medium [30]. Since formation of the E-lysis tunnel is limited to certain areas [32] the overall structure of the bacterium remains intact leaving behind an empty cell envelope that is called Bacterial Ghost. BGs share all antigenic properties of their viable counterparts as morphological structures like membrane proteins, pili and LPS remain unaltered. This feature makes BGs ideal candidate vaccines with inherent adjuvant properties as has been suggested repeatedly [12,21,22,25,29]. It has been shown that mucosal delivery of BG candidate vaccines is a favorable route of administration inducing both humoral and cellular immune response [27]. This enables needle-free application of the vaccine as well as frequent boosting. BG products are presented as freeze-dried powder and can be stored and shipped cold chain-independent. Activity of BG membrane associated ATPase did not decrease significantly after five years of storage as compared to freshly lyophilized BGs [14]. This is interpreted as proof that protein integrity within the cell envelope is maintained upon long-storage at room temperatures.

For production of BGs it is of vast importance to have tight control over the expression of lysis gene E. Bacteria harboring an E-lysis plasmid are grown to certain densities before E-lysis is induced. For production of BGs the use of a temperature-inducible P/O system is advantageous as it forgoes the addition of chemical inducers. To enable Bacterial growth at 35°C and E-lysis induction at elevated temperatures (>39°C) a modified temperature inducible λp₅₋c1857 P/O-system can be used [13].
The BG product itself is mostly free of both bacterial and plasmid DNA which is expelled during the process of E-lysis and withdrawn during product refinement. It has been reported that the residual DNA concentration in BG products is less than 1% of the initial value at E-lysis induction [1]. The production process of BGs may be further extended by the expression of staphylococcal nuclease A during bacterial growth. The enzyme is activated towards the end of the E-lysis process as a secondary kill gene and for DNA degradation [8]. Ultimately, BG products are completely inactivated using β-propiolactone (BPL). Inactivation is effected by alkylation of nucleic acids leaving any residual DNA non-reproducible [26]. The anticipated carcinogen BPL gradually hydrolyzes in aqueous environments to non-hazardous 3-hydroxypropionic acid.

*Shigella flexneri* is one the four *Shigella* species causing shigellosis, the others are *S. dysenteriae, S. boydii* and *S. sonnei* [24]. Shigellosis is characterized by severe diarrhea or dysentery accompanied by symptoms such as bloody stool, abdominal cramps and tenesmus. As of 1999 the worldwide number of shigellosis episodes has been estimated to about 165 million per year, the annual fatality rate due to shigellosis is approximately 1.1 million deaths per year [15]. *S. flexneri* holds responsible for 60% of the total episodes with *S. flexneri* 2a (Sf2a) being the predominant serotype [15]. Shigellae are highly contagious with infectious doses of as few as 100 cells for Sf2a [7]. In most cases Shigellae are transmitted by person-to-person or feacal-oral contact, rarely infection can be spread by contaminated food and water or even household flies [24]. Infections with Shigellae in almost all cases (99%) appear in regions with poor hygienic conditions and rudimentary health care (developing countries) [15]. In accordance with the WHO guideline (WHO/CDR/95.3) treatment of shigellosis focuses on proper re-hydration and administration of a suitable antibiotic. However, the propagation of resistance against a variety of antibiotics and antimicrobials is an issue [4]. Protective immunity can be reached by infection with wild type Shigellae; this immunity is serotype specific [24]. Development of an efficacious vaccine against Sf2a would be big step towards containing endemics of shigellosis especially in developing countries. In addition there is a market for such vaccine for the military and also travelers. Current vaccine approaches use e.g. live-attenuated or inactivated whole-cell vaccines; but to the date no candidate vaccine both efficient and safe has been presented [24].

Staphylococcal nuclease A (SNUC; EC 3.1.31.1) is the extracellular endonuclease of *Staphylococcus aureus* [28]. SNUC hydrolyzes both DNA and RNA leaving 3’ mono- and oligonucleotides. Its activity is unconditionally dependent on Ca$^{2+}$ ions [9]. The optimal concentration of Ca$^{2+}$ is interrelated with the pH; the predominantly suggested Ca$^{2+}$ concentration
for enzyme activation is 10 mM with a pH optimum in the range of 9 to 10 [5]. Mg^{2+} ions at a concentration of 1 mM have a stimulatory effect on DNA hydrolysis when supplemented alongside Ca^{2+} [5]. Overexpression of cloned SNUC in different bacteria results in intracellular activity and a significant loss of viability in the respective population [23]. Controlled expression of cloned SNUC has thus been suggested as a suicidal genetic element [23]. Co-expression of lysis gene E with staphylococcal nuclease A has been reported for Escherichia coli with greatly improved bacterial inactivation [8].

3.2 Materials and methods

3.2.1 Strain and plasmid

Shigella flexneri 2a (ATCC 700930) was obtained directly from ATCC (Manassas VA, USA). The cells were transformed with plasmid pGLN1c (see fig. 3.1) via electroporation. pGLN1c carries the lysis gene E under thermosensitive control of the λpR promoter as well as the IPTG-inducible gene coding for SNUC (lac-operon). For clone selection the plasmid furthermore has a Gentamicin resistance cassette. Clones were tested with respect to growth and E-lysis performance. Stocks were produced as 25% (v/v) glycerol stocks (1.8 ml) and stored at -80°C.

Plasmid construction: plasmid pGLN1c was derived from E-lysis plasmid pGLysivb [17] by inserting the staphylococcal nuclease A gene SNUC together with the Lac promoter A1/O4/O3 and the lacI² repressor for tight regulation of protein expression. A 2.1 kb fragment containing the functional SNUC cassette of plasmid pSNUCIQ3 [20] was generated by PCR amplification. Plasmid pSNUCIQ3 was used as template and primers 5pLNI and 3pLNI to introduce terminal SpeI restriction sites: sense (5pLNI) 5'-ACACTAGTCTTAAGCTCGAGCGGCCGCTAA-3', anti-sens (3pLNI) 5'- TAACTAGTCCGGCTAGCTTGGATTC TCAC-3'. The PCR condition for generating the PCR fragments were as follows: 30 cycles of amplification (45 sec at 94°C, 30 sec at 55°C and 60 sec at 72°C) were performed after 3 min of pre-denaturation at 94°C. The PCR fragment was cloned in the corresponding restriction site of lysis plasmid pGLysivb after linearization with SpeI. The insertion of the SNUC fragment in the correct orientation yielded into E-lysis/nuclease plasmid pGLN1c.

3.2.2 Production of BGs

Sf2a (pGLN1c) BGs were produced in 20.0 l low density batch fermentations using a 30 l Techfors-S fermenter (INFORS-HT, Bottmingen, Switzerland). Fermentations were per-
formed in complex Luria-Bertani medium (LB\(^+\)): 10.0 g/l tryptone (ROTH, Karlsruhe, Germany), 5.0 g/l yeast extract (ROTH), 10.0 g/l NaCl. Pre-cultures were grown at 35°C overnight in 4 x 500 ml LB\(^+\). All media were prepared with de-ionized water. After sterilization LB\(^+\) was supplemented with 10.0 g/l gentamicin stock (gentamicin sulfate\(^a\)) to a final concentration of 20.0 mg/l. Inoculation of the fermenter was done with 1.5 l pre-culture.

Figure 3.2 shows the timeline for a Sf2a (pGLNiC) BG production process. Bacteria grew for 60 min at 35°C, pH 7.20 and increasing aeration settings: air flow 0.6 to 0.8 vvm (12 to 16 l/min), stirring 120 to 300 rpm. IPTG (expression of SNUC) was added at a final concentration of 0.1 mM 30 min prior to E-lysis induction (LI). LI was effected by a temperature shift to 44°C, the timepoint of LI is referred to as 0 min. SNUC was activated at 90 min by a pH shift to 8.0 and addition of CaCl\(_2\) and MgCl\(_2\) at final concentrations of 10 mM and 1 mM, respectively. After activation of SNUC the fermentation was continued at the given conditions for another 180 min. Foaming during E-lysis phase was counteracted with automated addition of Antifoam A (SIGMA, St. Louis MO, USA).

Inactivation via β-propiolactone (BPL, Ferak, Berlin, Germany, 98.5% pharmaceutical grade) was done with a total amount of 0.75%/perthousandzero (v/v). Two doses of 7.5 ml BPL, each, were added to the fermenter via injection at 270 min and 300 min. The stirring rate was increased to 600 rpm for BPL treatment, the total application time was 60 min.

3.2.3 Sampling and analysis

Samples (25 ml) were taken throughout the whole fermentation process. The timepoints of sampling are marked with capital letters (A-M) in figure 3.2. Samples were analyzed with respect to the optical density at \(\lambda = 600\) nm (OD\(_{600}\)) and colony forming units (cfu). cfu determination was done on Plate Count Agar (ROTH) from appropriate dilution series in 0.85 (w/v) saline solution using a WASP spiral plater (DON WHITLEY SCIENTIFIC, Shipley, UK). Colony quantification was done with a ProtoCOL SR 92000 colony counter (SYNOPTICS LTD, Cambridge, UK); 50 µl samples were plated as quadruplets. E-lysis effectively kills bacteria which can be followed in the cfu data; the performance of a BG production process is judged by the lysis efficiency (LE).

\[
LE = \left(1 - \frac{\text{cfu (90 min)}}{\text{cfu (0 min)}}\right) \times 100\%
\]

Samples taken at 330 min and 360 min (30/60 min after the second BPL dose) were plated undiluted (50, 100, 200, 400 µl) to verify complete inactivation.

Real-time PCR analysis was performed for samples C, E, K-M to quantify the reduction
of DNA with an iCycler iQ™ RT-PCR Detection System and iQ™ SYBR green Supermix (Bio-Rad, Hercules CA, USA). Primers specific for the gentamicin resistance cassette on plasmid pGLNlc are GfwRT (5'-AGCGCGATGAATGTCTTACTACG-3’) and GrevRT (5’-TCCATGCGGGCTGCTCTTGAT-3’). Standards were prepared from known plasmid concentrations as 10^{-1} to 10^{-8} dilution series; standard curves with a correlation of R^2 < 0.998 were rejected.

3.2.4 BG harvesting

After inactivation with BPL the culture broth was harvested via mechanical separation in a disc stack centrifuge CTCI-06-107 (GEA WESTFALLA SEPARATOR, Oelde, Germany). The culture broth is driven out of the fermenter and into the rotor of the separator utilizing the head pressure in the vessel. The streams into and out of the rotor are controlled with membrane valves and balanced so that the supernatant is clear (OD_{600} < 0.1).

The whole 20 l culture broth were harvested in one cycle. Subsequently the rotor was removed from the separator and the pelletized BG product was collected under laminar flow. Washing of the BG product was done by repeated re-suspension/centrifugation cycles gradually reducing the re-suspension volume from 1,600 to 400 ml. After the final re-suspension the BG product was spread to 10 x 40 ml aliquots and frozen at -20°C/-80°C for lyophilization.

3.2.5 Sterility control

The lyophilized product was tested for sterility. 2 ml of re-suspended BGs (10 mg/ml) were prepared with sterile LBv (‘vegetarian’) medium (10.0 g/l soy peptone (Roth), 5.0 g/l yeast extract (Roth), 5.0 g/l NaCl). 2 * 200 µl of the suspension were prepared with Koch’s plating method in 20 ml sterile Plate Count Agar (Roth), each. 100 and 200 µl of the undiluted suspension were plated on Agar plates; all plates were incubated at 28°C for 24 h. 100/200 µl of the suspension were incubated in 5 ml sterile LBv, each, as enrichment cultures at 28°C for 24 h. 100/200 µl of the enrichment were plated and kept at 28°C for another 24 h. Total sterility of the product was verified by all tests being negative for viable cells.

3.2.6 Negative control

The fermentation setup as defined for production of Sf2a (pGLNlc) BGs was applied for S. flexneri 2a cells harboring the backbone plasmid pBBR1MCS-5 [16] without the cassettes for
Chapter 3 - Production of DNA-free Sf2a BGs

E-lysis and SNUC as a negative control (NC). The fermentation timeline was: inoculation at -60 min, IPTG (0.1 mM) addition at -30 min, LI at 0 min, addition of CaCl$_2$/MgCl$_2$ (10/1 mM) and pH = 8.0 at 30 min, BPL addition at 60 and 90 min. The process was continued for another 150 min after the second BPL addition. Samples were taken every 30 min and analyzed as described for the BG production process.

3.3 Results

3.3.1 Single Sf2a (pGLNiC) BG production run

The production process for Sf2a (pGLNiC) BGs described here is a low density process. Figure 3.3 shows a plot of viability parameters OD$_{600}$ and cfu vs. process time for the whole production process. After inoculation of the fermenter the cells resumed growth during the first 30 min (sample B). IPTG was added for SNUC expression what resulted in an apparent growth retardation as determined from the cfu while the OD$_{600}$ kept increasing (sample C). E-lysis was induced followed by an immediate notable drop in cfu. Calculating the E-lysis efficiency after 90 min (sample E) gives a value of LE = 99.97%. The number of viable cells has been decreased by more than three logs by E-lysis alone.

Immediately after sample E was taken the process conditions were changed for activation of the nuclease (pH = 8.0, addition of CaCl$_2$ and MgCl$_2$). During the first 90 min of SNUC activity (samples E to H) the cfu did not change much. In the following 90 min, however, the number of viable cells was further reduced significantly (samples H to K). This reduction again corresponds to an inactivation of more than three logs with respect to the end of E-lysis phase due to SNUC activity. Overall, the number of viable cells has been reduced by almost seven logs. The remaining surviving cells were in the following killed with BPL. In this fermentation run the first dose of BPL was sufficient (sample L).

RT-PCR was performed for samples C (LI), E (end of E-lysis/SNUC activation) and K/L/M (BPL: final inactivation) to substantiate the beneficial effect that SNUC has on the product properties besides enhanced inactivation. Figure 3.4 shows the curve for DNA concentrations after LI. The curve shape illustrates how DNA was gradually eliminated from the product over the whole process. The initial reduction during E-lysis phase was almost two logs (samples C to E). After SNUC activation the DNA concentration was further decreased over the next 180 min (samples E to K) by another two logs. Overall, at the end of the process (sample M) the DNA contents in the product has been diminished by 99.993% as compared to the timepoint of LI. For samples K to M the DNA concentrations approached...
the detection limit of 0.02 ng/ml DNA for RT-PCR amplification of the gentamicin resistance cassette as reported by Haidinger et al. [8].

3.3.2 Consistency study for Sf2a (pGLNIc) BG production

In the consistency study (CS) the production process for Sf2a (pGLNIc) BGs was repeated five times under the exact same conditions in order to show that all relevant parameters can be held in narrow windows. Relevant parameters in this context are the number of viable cells at LI (cfu, C), the E-lysis efficiency after 90 min (cfu, C/E), the degree of further inactivation due to SNUC activity (cfu, K) and the efficacy of BPL treatment. Also, emphasis was put on the fact that DNA is gradually eliminated from the product due to E-lysis (RT-PCR, C/E) and SNUC activity (RT-PCR, M). The data of all five runs as well as the average values for the individual parameters are presented in table 3.1.

LEs of at least 99.9% were accomplished in all five runs. In fact, the mean LE (99.94% ± 0.02) was almost half a log higher. The combined inactivation performance of E-lysis and SNUC activity reached almost six logs and the remaining surviving bacteria were effectively killed by BPL-treatment within 60 min resulting in a bioburden free product (no surviving cells detected in sample M). A graph summarizing the inactivation levels as determined by the cfu for the relevant timepoints (samples C/E/K/M) is given in figure 3.5 (a). Figure 3.5 (b) shows the average reduction of DNA in the product during the consistency study. At the end of the process (sample M) the measured DNA concentration was at the detection limit of RT-PCR for all five runs. Sterility was confirmed in all cases for the lyophilized BG product.

3.3.3 Negative control

Two fermentations with Sf2a carrying the plasmid backbone pBBR1MCS-5 were performed as negative control (NC runs 01 and 02). The cfu at 0 min was 8.03 and 3.63 · 10^8 cells per ml, respectively. The course of cell viability for both NC runs is shown in figure 3.6. Neither events related to gene expression of protein E or SNUC (IPTG, T-shift) nor SNUC activation (CaCl2/MgCl2, pH = 8.0) had an inactivating effect on the culture. The cfu values at 60 min rather increased to 1.76 · 10^9 (NC 01) and 5.51 · 10^8 (NC 02) cells per ml. The first addition of BPL at 60 min resulted in an initial decrease of cell viability. Significant loss of viability was detected after the second BPL addition (90 min). Until the end of the fermentations the cell viability dropped in runs NC 01 and 02 to 1.46 · 10^5 and 1.07 · 10^5 cells per ml, respectively. This corresponds to BPL-related inactivation of almost four logs
for both runs. The administered amount of BPL, however, was insufficient for complete inactivation of the cultures that were not previously affected by E-lysis and SNUC activity.

The curves for the DNA concentration in samples taken from runs NC 01 and 02 as determined by RT-PCR are plotted in figure 3.6 as well. With regard to the whole fermentation the DNA contents did not change significantly.

3.4 Discussion

A production process for Sf2a (pGLNiC) BGs was presented. The targeted combination of the three approaches E-lysis, staphylococcal nuclease A activity and treatment with β-propiolactone lead to a product with the following major features: it consisted of more than 99.9% BGs, the concentration of residual DNA was at the detection limit of real-time PCR and the product was bioburden free before lyophilization. In a consistency study all results proved to be reproducible.

E-lysis using plasmid pGLNiC was very effective in Sf2a as the mean LE in the consistency study was LE = 99.94 ± 0.02% after 90 min. The DNA concentration in the BG product was reduced by an average of two logs during E-lysis phase as the cytoplasm was expelled. The residual concentration of DNA after E-lysis was in the magnitude of 1% as compared to the timepoint of E-lysis induction. This confirmed previous findings with E-lysis of E. coli [1]. After activation of SNUC the DNA concentration was further reduced by another 2.5 logs which corresponded to an overall reduction of the DNA contents by 99.994%. As the DNA concentration in the BG pellet could be reduced to the detection limit of RT-PCR the product was essentially DNA free. In addition to the degradation of DNA SNUC activity effected an enhanced decline of viability. With respect to the end of E-lysis phase the number of surviving bacteria was further reduced by almost three logs. Animal studies with different BG candidate vaccines reported that after lyophilization no surviving bacteria were detected in freeze-dried BG products [11, 18, 20]. Yet, for safety reasons it is mandatory to completely inactivate the BG suspension before lyophilization. Inactivation was accomplished by treatment with 0.75%/perthousandzero (v/v) BPL administered directly to the culture broth in two equal doses with a delay of 30 min. In four out of five cases the first dose was already sufficient for total inactivation. It was anticipated that due to continued stirring and aeration during BPL treatment surviving bacteria could be trapped in droplets at the lid or walls of the vessel while the first dose of BPL was administered and backdrop later. Since
BPL hydrolyzes with time in aqueous environments those bacteria could be unaffected by the initial BPL administration which made a second dose inevitable. During the consistency study all relevant parameters could be held in narrow windows underlining the stability of the production process.

In both runs of the negative control fermentation viability of the culture was unaffected by both the T-shift to 44°C and the change of conditions for SNUC activation (pH 8.0, addition of CaCl₂/MgCl₂). Addition of BPL reduced the viability of the culture by four logs over the course of 180 min but was insufficient to fully inactivate all bacteria in the culture. Over the course of the fermentations no reduction of DNA was detected. This indicates that the cells were partly inactivated but not disintegrated by BPL as the DNA remains associated with the bacteria. The negative control fermentations confirm that the desired product properties were only achieved by combined performance of E-lysis, SNUC and BPL.

This production process for Sf2a (pGLN1c) BGs is short, incomplex and does not incorporate cost-intensive methods. The product has all beneficial features attributed to BG vaccines such as self-adjuvant properties and maintained antigenic structures. The fact that the product is essentially DNA free eliminates the risk of horizontal gene transfer of pathogenicity islands or antibiotic resistance genes. The lyophilisate can be shipped and stored at room temperature without a loss of efficacy and facilitates needle-free administration.
3.5 Figures and tables

Figure 3.1: Plasmid map for E-lysis plasmid pGLNiC


Figure 3.2: Timeline and sampling points for the Sf2a (pGLNiC) BG production process

Consecutive sampling points are marked with capital letters. Events: 0.1 mM IPTG for expression of SNUC, T-shift 35 to 44°C for E-lysis induction, 10 mM CaCl2/1 mM MgCl2/pH 8.0 for SNUC activation, BPL addition (total of 0.75%/perthousand/v/v) in two equal doses. Harvesting is done with a separator.
Figure 3.3: Viability curve for a single Sf2a (pGLN1c) BG fermentation

Capital letters mark relevant samples. Events immediately after the corresponding sample is taken are B: addition of 0.1 mM IPTG, C: T-shift (35 to 44°C) for E-lysis induction, E: pH = 8.0, addition of 10 mM CaCl₂/1 mM MgCl₂ for SNUC activation, K: 1st dose of BPL (7.5 ml), L: 2nd dose of BPL (7.5 ml). Since the cfu data is plotted on a log axis the event of no surviving cells detected (no surv.) is plotted at 1.0E-01.
Figure 3.4: Reduction of DNA contents in the product during a single Sf2a (pGLN1c) BG fermentation

E: pH = 8.0, addition of 10 mM CaCl₂/1 mM MgCl₂ for SNUC activation, K: 1st dose of BPL (7.5 ml), L: 2nd dose of BPL (7.5 ml). The DNA concentration in the BG product is detected by real-time PCR. The detection limit of RT-PCR (det. lim.) is DNA concentrations < 0.02 ng/ml as indicated by the dashed line [8].
Data for the individual runs (CS)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>E</th>
<th>K</th>
<th>LE (90 min)</th>
<th>DNA (ng/ml)</th>
<th>BPL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS run 01</td>
<td>1.29E+09</td>
<td>6.77E+05</td>
<td>1.16E+03</td>
<td>99.95%</td>
<td>670</td>
<td>14</td>
</tr>
<tr>
<td>CS run 02</td>
<td>8.95E+08</td>
<td>2.92E+05</td>
<td>1.46E+03</td>
<td>99.97%</td>
<td>220</td>
<td>1.6</td>
</tr>
<tr>
<td>CS run 03</td>
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Average data (CS)

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Table 3.1: Data for the consistency study of Sf2a (pGLNIc) BG production

Relevant parameters are: cfu and DNA contents at E-lysis induction (sample C, T-shift 35 to 44°C), cfu and DNA contents at SNUC activation (sample E, pH = 8.0, addition of 10 mM CaCl2/1 mM MgCl2), cfu before 1st BPL addition (sample K, 7.5 ml BPL), E-lysis efficiency at 90 min, DNA contents at the end of the process (sample M). * BPL treatment: successful after 1st (L) or 2nd addition (M). nd: not determined.
Figure 3.5: Consistency study for Sf2a (pGLNiC) BG production: cfu and DNA contents

(a) Average viability in the CS as determined from cfu data - since viability is plotted on a log axis the event of no surviving cells detected (no surv.) is displayed at 1.0E-01. (b) Average DNA concentration in the CS as detected by real-time PCR. The detection limit of RT-PCR (det. lim.) is DNA concentrations < 0.02 ng/ml as indicated by the dashed line [8]. C: E-lysis induction (T-shift 35 to 44°C), E: SNUC activation (pH = 8.0, addition of 10 mM CaCl₂/1 mM MgCl₂), K: 1st dose of BPL, M: end of process
Figure 3.6: **Negative control for Sf2a (pGLNIc) BG production: cfu and DNA contents**

Events are marked with lower-case letters referring to the events during Sf2a (pGLNIc) BG production (capital letters at corresponding sample). b: addition of 0.1 mM IPTG, c: T-shift 35 to 44°C, e: pH = 8.0, addition of 10 mM CaCl$_2$/1 mM MgCl$_2$, k: 1$^{st}$ addition of 7.5 ml BPL, k: 2$^{nd}$ addition of 7.5 ml BPL.
Chapter 3 - Production of DNA-free Sf2a BGs

3.6 References


4 Bacterial Ghost production: 
\(\beta\)-propiolactone inactivation of non-lysed bacteria

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Bacterial Ghosts (BGs) are produced from E-lysis in Gram-negative bacteria and have been suggested as candidate vaccines or drug delivery vehicles. Final inactivation of non-lysed bacteria in BG products by treatment with \(\beta\)-propiolactone (BPL) was investigated. For low density batch fermentations (\(\leq 10^9\) cells per ml) of \textit{Shigella flexneri} 2a (Sf2a) and \textit{Escherichia coli} Nissle 1917 (EcN) BGs where the culture broth was ten-fold concentrated before inactivation the BPL concentration required for killing all surviving bacteria within 60 min was 0.075\% (v/v) when applied at 44\(^\circ\)C. The minimal amount of BPL to achieve total inactivation was shown to be dependent on the degree to which free DNA that was expelled during E-lysis could be removed from the broth. For medium density batch fermentations (\(\leq 10^{10}\) cells per ml) of EcN BGs where the culture broth was washed by dia-filtration prior to concentration 0.50\% (v/v) BPL were sufficient for inactivation of the BG product.
**List of abbreviations**


**4.1 Introduction**

Bacterial Ghosts (BGs) have been suggested as potent new candidate vaccines [19, 33, 34]. BGs are empty cell envelopes that can be produced from various Gram-negative bacteria by E-lysis. E-lysis is characterized by the controlled expression of bacteriophage \(\phi X174\)-derived lysis protein E that is co-translationally incorporated into the inner membrane of the bacterium [2]. Protein E does not exhibit any known enzymatic function [21], moreover it is known to oligomerize and induce fusion of the inner and outer membrane in Gram-negative bacteria resulting in a typical trans-membrane tunnel structure [3]. It has been shown that this E-lysis tunnel is predominantly found at potential cell division sites [40, 41] and its formation further requires the cell autolytic system to be functional [18, 38] as E-lysis is effective in actively dividing cells. Once fusion of the membranes is induced the trans-membrane tunnel structure is established driven by the osmotic pressure difference between the cytoplasm and the environment [41]. During the process the periplasmic space is sealed while the cytoplasmic contents (including DNA) is expelled leaving behind the empty cell envelope [39]. Since E-lysis leaves the overall morphology of the cell envelope intact BGs resemble the bacterium they are derived from. Antigenic structures such as LPS, membrane proteins, pili etc. are conserved during the process making BGs potent self-adjuvanting candidate vaccines [31]. Prior to E-lysis foreign target antigens can be integrated into the inner or outer membrane of the bacterium via fusion to membrane anchor sequences [10, 34] or secreted to the periplasmic space [35]. By this means the range of application for BGs as vaccines can be extended significantly. The concept of using native as well as extended BGs as candidate vaccines and drug delivery vehicles has been reviewed [15, 16, 24, 25, 28, 37].

Production of safe inactivated vaccines requires a reliable inactivation method. Inactivation, i.e. killing, can be achieved by treatment with heat, irradiation or chemical agents [5, 22, 36]. Any inactivation strategy should be effective and safe while antigenicity of the vaccine should remain unaffected. While heat generally causes notable denaturation antigenicity has been reported to be well conserved after chemical treatment with β-propiolactone [36]. β-propiolactone (BPL) is a water-soluble organic compound with
alkylating properties. At low concentrations it is known to attack and alter the structure of nucleic acids, mainly guanine, inducing nicks and cross-links in the double helical structure of the DNA strand [29]. These alterations prevent DNA transcription and replication and thus effectively kill viruses and bacteria [7, 8, 17]. BPL is toxic and due to its action on DNA has been classified ‘reasonably anticipated to be a human carcinogen’ by the US Department of Health and Human Services [27]. However, in an aqueous environment BPL hydrolyzes forming non-hazardous 3-hydroxypropionic acid (3-HPA). The time required to achieve full hydrolysis thereby depends on the prevalent temperature; at 37°C degradation is complete after 3 h [29]. Also, the inactivation performance of BPL is temperature dependent. Kamaraj et al. have investigated the inactivation kinetics of Bovine herpes virus-1 (BHV-1) using different BPL concentrations at 4 and 37°C. Independent of the temperature they found direct proportionality between the applied concentration of BPL and the rate of inactivation [13]. At BPL concentrations ranging from 4 to 0.4% (v/v) at 4°C the virus was completely inactivated in a matter of 8 to 54 h while using equal amounts of BPL at 37°C complete inactivation was a matter of only 40 to 480 min, respectively [13]. Presuming that complete degradation of BPL is guaranteed its use during the production process of pharmaceuticals is not an issue which is why BPL is widely used to inactivate medicinal preparations such as e.g. blood plasma and also vaccines. Plotkin lists a variety of commercial rabies vaccines that are BPL-inactivated [30]. Many other examples have been reported for vaccines featuring virus-inactivation via BPL [4, 13, 14]. BPL has not only been used for virus inactivation [7]; just recently pre-clinical studies for a BPL-inactivated whole cell vaccine against Streptococcus pneumoniae have been reported. For killing the washed cells were circulated for 24 h at 4°C with 0.25% (v/v) BPL and subsequently incubated at 37°C for two more h to achieve BPL hydrolysis [17].

BGs have been used as inactivated candidate vaccines. Animal studies have been conducted with BG products that were not inactivated before lyophilization. The freeze-dried products were reported bioburden free [9, 20, 23]. However, as E-lysis is a biological process it never affects 100% of the cells an additional inactivation step is necessary for safety reasons of handling. For this purpose the use of BPL at low concentrations was introduced. BGs and surviving cells only have a low content of residual DNA of less than 1% as compared to viable cultures [1]. The other 99% of DNA have been expelled from the envelopes and exist as free DNA in the medium. In order to minimize the amount of BPL it is favorable to eliminate as much of the free DNA as possible from the product suspension prior to BPL addition. The downstream process for recovery of BGs was optimized with regard to this
requirement. Data presented in this work aims to define a minimal but sufficient amount of BPL required for reproducible total inactivation of BG products within a time window of 60 min for complete inactivation.

4.2 Materials and methods

4.2.1 Strains and plasmids

The inactivation studies using BPL were conducted with BGs derived from enteropathogen Shigella flexneri 2a (Sf2a) and probiotic Escherichia coli strain Nissle 1917 (EcN). Sf2a (ATCC 700930) was obtained from ATCC (Manassas VA, USA) while EcNΔ was provided courtesy of ARDEYPHARM (Herdecke, Germany). EcNΔ is devoid of two cryptic plasmids protecting the native strain against mobile genetic elements [32]. Strains Sf2a and EcNΔ were transformed with E-lysis plasmids pGLys36.1 and pGLysivb, respectively (plasmid maps see fig. 4.1). Plasmid pGLys36.1 has been described previously [11]. Plasmid pGLysivb is derived from pGLys36.1 by exchanging the lysis gene E with the Eivb fusion gene. The fusion of gene E and the invivo-biotinylation (ivb) sequence was cut from plasmid pFNEivb2 [26] using restriction enzyme XbaI. The 500 bp fragment carrying Eivb was cloned into the 6.2 kb fragment of pGLys36.1 obtained after digesting pGLys36.1 with XbaI. The insertion of the Eivb fragment in the correct orientation yielded into lysis plasmid pGLysivb. In addition to a gentamicin resistance cassette for clone selectivity both E-lysis plasmids are equipped with a modified temperature-inducible λpR-cl857 P/O system [12] for controlled expression of gene E. This modified version tightly represses gene expression at the bacterial growth temperature of 35°C while gene expression is induced by T-shifts to above 39°C.

Transformation of Sf2a with pGLys36.1 as well as EcNΔ with pGLysivb was done via electroporation. Successfully transformed clones were tested and selected with respect to growth and E-lysis. Working stocks of the chosen clones were prepared as 1.8 ml 25% (v/v) glycerol stocks and kept at -80°C.

4.2.2 Production of BGs

All BGs were produced as 20.0 l batch fermentations in a 30 l Techfors-S fermenter (INFORS-HT, Bottmingen, Switzerland). Low density Sf2a BG fermentations were conducted in complex EdOaks medium (EO, by Dr. Edwin V. Oaks, Walter Reed Army Institute of Research, Silver Springs MD, USA): 5.0 g/l yeast extract (BD, Franklin Lakes NJ, USA),
1.0 g/l dextrose (BD), 1.5 g/l vegetable peptone (OXOID, Hampshire, UK), 3.5 g/l NaCl. Pre-cultures (4 x 500 ml) were grown in two-fold concentrated, buffered EO⁺(2x): 10.0 g/l yeast extract (BD), 2.0 g/l dextrose (BD), 3.0 g/l vegetable peptone (OXOID), 7.0 g/l NaCl, 2.64 g/l KH₂PO₄, 7.36 g/l K₂HPO₄. Low density EcN BG fermentations as well as corresponding 4 x 500 ml pre-cultures were done in animal-protein free (‘vegetarian’) low salt Luria-Bertani medium LBv: 10.0 g/l soy peptone (ROTH, Karlsruhe, Germany), 5.0 g/l yeast extract (ROTH), 5.0 g/l NaCl. Medium density fermentations of EcN BG were done in a defined minimal medium according to DeLisa et al. [6]. The composition is: 22.00 g/l C₆H₁₂O₆ · H₂O, 13.30 g/l KH₂PO₄, 4.00 g/l (NH₄)₂HPO₄, 1.70 g/l C₆H₈O₇ (citric acid), 1.20 g/l MgSO₄ · 7 H₂O, 0.1000 g/l C₆H₈O₇ · Fe, 0.0084 g/l EDTA, 0.0130 g/l Zn(CH₃COO)₂ · 2 H₂O, 0.0025 g/l CoCl₂ · 6 H₂O, 0.0150 g/l MnCl₂ · 4 H₂O, 0.0012 g/l CuCl₂ · 2 H₂O, 0.0030 g/l H₃BO₃, 0.0025 g/l Na₂MoO₄ · 2 H₂O, 0.0045 g/l thiamine HCl, 1.00 g/l poly-propylene glycol (PPG - SIGMA, St. Louis MO, USA). Pre-cultures (3 x 500 ml) were grown in the same medium (DeLisa, no PPG) with reduced glucose concentration of 8.0 g/l. All media were prepared with de-ionized water (dH₂O).

BG fermentations divide into three stages: growth phase, E-lysis phase and downstream processing. The growth phase aims for unlimited growth at 35°C, pH 7.20 and sufficient aeration. For low density fermentations the aeration rate was 0.6 to 0.8 vvm compressed air with a maximal stirring rate of 600 rpm. For medium density fermentations the aeration rate was 1.0 to 2.0 vvm compressed air with constant stirring at 1,000 rpm. The growth phase lasted 90 to 120 min (low density) and 270 min (medium density), respectively. Growth was followed in the optical density at λ = 600 nm (OD₆₀₀) as well as with the determination of colony forming units (cfu). E-lysis phase started with the induction of protein E expression through a T-shift to 42 (EcN) or 44°C (Sf2a) and continued for 90/120 min (Sf2a/EcN).

The point of E-lysis induction (LI) is referred to as timepoint zero (0 min). As E-lysis proceeds the cfu drops, at the end of E-lysis phase (120 min) the lysis efficiency (LE) is determined from the cfu as follows:

\[
LE = \left(1 - \frac{\text{cfu (120 min)}}{\text{cfu (0 min)}}\right) \cdot 100\%
\]

4.2.3 cfu determination

To determine viable cell counts via cfu appropriate dilution series of the samples were prepared in 0.85% (w/v) saline solution. 50 µl of the diluted samples were plated on Plate Count Agar ROTH using a WASP spiral plater (DON WHITLEY SCIENTIFIC, Shipley, UK).
Colony quantification was done with a ProtoCOL SR 92000 colony counter (Synoptics Ltd, Cambridge, UK). For every diluted sample the cfu determination was performed as quadruplet. During BPL treatment for samples where no surviving cells were expected a series of 50, 100, 200 and 400 µl of the pure suspension was plated. For sterility testing of inactivated BG products 3 x 400 µl of undiluted concentrate were plated on agar plates. Enrichment cultures of 200/400 µl undiluted concentrate in 5 ml LBv were incubated at 35 °C for 48 h. 3 x 400 µl of enrichment culture were subsequently plated on agar plates. Agar plates were incubated 24 h at 35°C.

4.2.4 Downstream processing

Downstream processing featured tangential flow filtration (TFF). TFF was done using a CFP-2-E-8A hollow fiber module (GE Healthcare, Chalfont St. Giles, UK); membrane area: 0.36 m²; cut-off: 0.2 µm. The BG suspension was withdrawn from the bottom of the fermenter vessel, passed through the TFF module with a pump rate of 9.0 l/min using a Flowmaster FMT 300 peristaltic pump (Ismatec, Glattbruck, Switzerland) and re-circulated to the vessel overhead. All connective tubing was ID 16 mm Rotilabo (Roth) silicone tubes. The pressure loss Δp in the TFF module was monitored with a digital manometer GDH 13 AN (Greisinger, Regenstauf, Germany) and adjusted (300 to 600 mbar) by fastening/loosening a hose clamp downstream of the module.

Directly after E-lysis phase the culture broth was concentrated by TFF to a minimum volume of approximately 500 to 800 ml. The concentrate was then flushed from the vessel and TFF system into a sterile container (stirred glass bottle) with sterile dH₂O and filled up to a total volume of 2.0 l. The 2.0 l concentrated product suspension (concentration factor 10) were then treated with BPL.

E-lysis phase of medium density batch fermentations (EcN in DeLisa medium) incorporated a washin step. The culture broth volume was exchanged once with 20.0 l sterile dH₂O by dia-filtration (DF) in the same TFF module as for harvesting. The liquid volume in the vessel was held constant with a fresh water stream compensating the permeate flux across the TFF membrane.

4.2.5 BPL treatment

Treatment with β-propiolactone (BPL, 98.5% pharmaceutical grade, Ferak, Berlin, Germany) was done in the 2.0 l concentrate. Designated BPL concentrations were defined as %₀(v/v₀) where v₀ referred to the total fermentation volume of 20.0 l. The total amount
Chapter 4 - BG production: BPL-inactivation of non-lysed bacteria

of BPL was split and administered in two equal doses with a delay of 30 min. For screening experiments the concentrated product was divided into 200 ml aliquots in sterile 500 ml Erlenmeyer flasks equipped with magnetic stirrers. Flasks were stirred at 300 rpm in waterbaths at the designated temperature for 15 min prior to the first addition of BPL.

4.2.6 DNA concentration measurements

DNA concentration in the supernatant of the culture broth was determined photometrically. Extinction measurements were performed at $\lambda = 260/280$ nm in an Infinite 200 Mircroplate Reader (Tecan, Männedorf, Switzerland) using UV-transparent well plates. The DNA concentration is estimated ($\mu$g/ml):

$$c_{\text{DNA}} = E_{260} \cdot 50$$

For reliable results the ratio of $E_{260}:E_{280}$ should be $> 1.8$

4.3 Results

Figure 4.2 shows the viability curve of a low density fermentation ($\leq 10^9$ cells per ml at LI) of Sf2a (pGLys36.1) BGs in EO medium. After 90 min of growth phase the T-shift for LI (35 to 44°C) was performed resulting in an immediate drop of both viability parameters OD$_{600}$ and cfu. At the end of E-lysis phase the number of viable cells was decreased by 99.91%. The BG product was subsequently ten-fold concentrated via TFF.

Screening experiments varying both temperature and $\beta$-propiolactone concentration were performed with BG concentrate to define the most suitable combination of those two parameters for total inactivation of BG products. The total amount of BPL as designated by the final concentration was split and delivered in two equal doses with a delay of 30 min. Samples were taken for cfu determination before each dose of BPL was given (0/30 min). Screening experiments were performed as given in tables 4.1 and 4.2.
4.3.1 Screening experiments with Sf2a (pGLys36.1) BGs

Three low density fermentation runs for Sf2a (pGLys36.1) BGs were performed in complex EO medium with an average yield of $8.20 \cdot 10^8$ cells per ml ($\sigma = 6.24 \cdot 10^7$) and an average LE of 99.940% ($\sigma = 0.026$). Screening experiments as described in table 4.1 were performed for each fermentation. Figure 4.3 depicts the results for the three runs separately for both application temperatures 44°C (a) and 20°C (b) as cfu vs. application time. BPL doses were given at 0 and 30 min. The cfu was plotted on a logarithmic scale so the event ‘no surviving bacteria detected’ (0 cells/ml) could not be displayed. Instead, this event (no surv.) was plotted at 1.0E+00 on the log scale. The decline in viable cells over time was fitted to an exponential function.

In the experiments performed at 44°C all three different BPL concentrations of 0.050, 0.075 and 0.100% (v/v) (B/C/D) caused complete inactivation in every case within 60 min. Concentrations of 0.100% (v/v) BPL (D) were sufficient for complete inactivation after 30 min in two out of three cases. The control with no BPL addition (A) showed a slight decrease in cfu over time at 44°C. For the experiments conducted at 20°C only a concentration of 0.100% (v/v) BPL (H) was sufficient to repeatedly inactivate all surviving bacteria. In all three runs it took an application time of 90 min to accomplish inactivation. BPL concentrations of 0.075% (v/v) effected total inactivation after 120 min in two out of three cases (G), lower concentrations failed to fully inactivate the BG product in the observed timeframe (F). The control with no BPL addition (E) showed that at 20°C the number of surviving Sf2a (pGLys36.1) was stagnant over the 120 min period.

4.3.2 Screening experiments with EcN (pGLysivb) BGs

Screening experiments following the scheme as given in table 4.1 were repeated for BG products obtained from three different low density EcN (pGLysivb) batch fermentations in complex LBv medium. For production of EcN (pGLysivb) BGs both growth and E-lysis phase were extended to 120 min, E-lysis was induced by a T-shift from 35 to 42°C. The results of the screening experiments were very similar to the results shown for Sf2a (pGLys36.1) BGs except that at 44°C 0.050% (v/v) BPL (B) failed to achieve total inactivation of the EcN (pGLysivb) BG product in one out of three cases. Further experiments with EcN (pGLysivb) BGs were conducted at 42°C following the scheme given in table 4.2. Again, three fermentations were performed yielding $9.72 \cdot 10^8$ cells per ml ($\sigma = 2.76 \cdot 10^8$), the average LE was 99.993% ($\sigma = 0.003$). Results for the three runs are shown in figure 4.4. All
three applied concentrations of BPL (0.075/0.100/0.150\%\% (v/v), B/C/D) were sufficient for complete inactivation at 42°C within 60 min. The control with no BPL addition (D) revealed that non-lysed EcN (pGLysivb) are able to resume growth at 42°C.

Detailed results for all screening experiments with Sf2a (pGLys36.1) and EcN (pGLysivb) BGs are summarized in table 4.3.

4.3.3 BPL treatment for medium density fermentations of EcN (pGLysivb) BGs

A medium density batch process in minimal medium (≤ 10^{10} cells per ml at LI) has been developed for EcN (pGLysivb) BG production. The cellular yield during the 270 min growth phase before LI was increased by the order of a magnitude as compared to the low density approach. Figure 4.5 includes a viability curve (cfu vs. process time) for such medium density process focusing on a timeframe of 120 min before and after LI. During E-lysis phase the culture broth was washed with 20.0 l dH\textsubscript{2}O via DF. A total of nine medium density fermentation runs for EcN (pGLysivb) BGs (cfu: 7.35 \cdot 10^9 \pm 1.23 \cdot 10^9 cells per ml, LE: 99.782 \pm 0.023\%) were performed. Inactivation of the whole 2.0 l concentrate was done with 0.50\%\% (v/v) BPL administered in equal doses (2 x 5.0 ml) with a 30 min delay. No surviving bacteria were detected after BPL treatment.

4.4 Discussion

During E-lysis the cytoplasmic contents and with it the DNA is expelled from the bacterial envelope. At the end of E-lysis this leaves > 99\% of the total amount of DNA as free DNA in the medium. In the procedure described here the culture broth at the end of E-lysis was 10-fold concentrated by TFF. By this measure 90\% of the medium and therein contained free DNA were withdrawn before BPL treatment. This reduced the total amount of DNA in the concentrate and helped to avoid side-reaction of BPL with free DNA. In previous low density BG productions BPL was added directly to the 20.0 l untreated culture broth at the end of E-lysis phase at 44°C in two equal doses. For inactivation of all surviving bacteria in a 60 min window BPL minimal concentrations of 0.75\%\% (v/v) were necessary (15 ml BPL vs. 20.0 l).

Screening experiments with Sf2a (pGLys36.1) and EcN (pGLysivb) BG products that were 10-fold concentrated before BPL treatment demonstrated that at 44°C BPL concentrations of 0.075\%\% (v/v) were sufficient for complete inactivation of non-lysed bacteria within 60 min. Since the temperature for E-lysis induction in EcN (pGLysivb) was 42°C the results
were additionally verified for application at 42°C for this BG product. The findings substantiate that reducing the amount of free DNA before BPL treatment directly impacts the minimal amount of BPL required for inactivation. For low density fermentations of Sf2a (pGLys36.1) and EcN (pGLysivb) BGs the BPL concentration could be reduced by the same factor (10) as the broth had been concentrated. It should be mentioned that the total amount of BPL was always calculated with respect to the initial total fermentation volume $v_f$ of 20.0 l, i.e. for inactivation of 2.0 l concentrate with 0.075%/perthousand zero (v/v) a total of 1.5 ml BPL were applied. At lower temperatures (20°C) the tested amounts of BPL were insufficient for total inactivation within 60 min for both types of BG tested. This is comprehensible since the rate of inactivation for BPL decreases with decreasing temperatures.

For medium density batch fermentations of EcN (pGLysivb) BGs in minimal medium the yield was increased by the factor 10 while values for LE were similar. Consequently at the end of E-lysis phase both the concentration of free DNA in the medium and the number of surviving bacteria are ten-fold higher, too. If the broth was concentrated as described for the low density process also a ten-fold higher BPL concentration (0.75%/perthousandzero (v/v)) would be necessary for inactivation. However, the process strategy for medium density fermentations incorporated a washing step during E-lysis phase. The culture broth volume was exchanged once with dH$_2$O by dia-filtration. Thereby significant amounts of the DNA expelled during E-lysis were withdrawn from the broth even before concentration. Figure 4.5 depicts how the measured DNA concentration in the culture broth supernatant escalated within 30 min after LI. This effect was retarded over the following 30 min and eventually reversed during the second half of E-lysis phase. No experimental results were available to determine how much DNA was actually removed from the broth by DF. Approximation with an exponential model (light blue curve in fig. 4.5, calculus not shown) suggested that the reduction was as much as 50%. This would require a BPL concentration of 0.375%/perthousandzero (v/v) after concentration. To assure complete inactivation the actual BPL concentration was raised to 0.50%/perthousandzero (v/v) for treatment EcN (pGLysivb) BG products obtained from medium density fermentations. This was sufficient for total inactivation of surviving bacteria in all nine fermentation runs performed.

The amount of BPL for required inactivation of BG products was shown to be essentially dependent on two parameters, i.e. the cell density and the degree to which free DNA could be eliminated from the culture broth prior to BPL treatment. Presuming similar values for LE, without further treatment the required amount of BPL is linear with the cell density at LI. In case washing or concentration steps are performed prior to BPL treatment the amount
of BPL can be reduced by the magnitude to which free DNA could be eliminated from the culture broth. In order to make accurate predictions for the minimal amount of BPL it is therefore important that the amount of free DNA in the broth can be reliably estimated.
4.5 Figures and tables

Figure 4.1: Plasmid maps for E-lysis plasmids pGLysivb and pGLys36.1


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</table>

Table 4.1: Setup 1 BPL screening

Table 4.2: Setup 2 BPL screening

Setup for the screening experiments. Capital letters define the conditions for individual experiments with temperature and BPL-concentration.
Chapter 4 - BG production: BPL-inactivation of non-lysed bacteria

Figure 4.2: Viability curve for a batch fermentation of Sf2a (pGLys36.1) BGs in EO medium

Viability data as obtain from OD$_{600}$ and cfu determination for a 20.0 l batch fermentation of Sf2a (pGLys36.1) BGs in EO medium. LI: E-lysis induction by T-shift (35 to 44°C).
Figure 4.3: Results for the BPL screening experiments with Sf2a at 20/44°C

BPL treatment of Sf2a BG product, setup 1. (a) BPL treatment at 44°C. (b) BPL treatment at 20°C. A/E: 0.000% (v/v). B/F: 0.050% (v/v). C/G: 0.075% (v/v). D/H: 0.100% (v/v). The event ‘no surviving bacteria detected’ (no surv.) is plotted at 1.0E+00. Data was fitted to an exponential model in MS Excel.
Figure 4.4: Results for the BPL screening experiments with EcN at 42°C

BPL treatment of EcN BG product, setup 2, at 44°C. A: 0.000% (v/v). B: 0.075% (v/v). C: 0.100% (v/v). D: 0.150% (v/v). The event no surviving bacteria detected (no surv.) is plotted at 1.0E+00. Data was fitted to an exponential model in MS Excel.
Figure 4.5: Viability and free DNA for a batch fermentation with EcN (pGLysivb) BG in DeLisa medium

Viability data as obtained from cfu determinations 120 min before and after LI. Free DNA in the culture broth supernatant was measured photometrically (DNA meas). An exponential model was included to estimate the amount of free DNA without purging by dia-filtration (DNA pred). LI: E-lysis induction by T-shift (35 to 42°C)
### Chapter 4 - BG production: BPL-inactivation of non-lysed bacteria

**Screening with Sfa - setup 1**

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**Screening with EcN - setup 2**

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**Table 4.3: cfu data obtained from the BPL screening experiments**

Results for cfu determinations during BPL screening experiments with Sf2a (pGLys36.1) and EcN (pGLysivb) BGs obtained from low density fermentation in complex medium. Conditions (A-H) as defined in tables 4.1 and 4.2. *: Plates overgrown due to improper dilution. nd: not determined.
4.6 References


Chapter 4 - BG production: BPL-inactivation of non-lysed bacteria


Process Development for Industrial Scale BG Production


5 Real-time assessment of E-lysis performance in *Escherichia coli* through flow cytometry

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Flow cytometry (FCM) is a tool for the detection of single cell properties in a suspension. FCM can be used for analysis of single parameters as well as for multiparameter assessment. Using FCM to evaluate formation of Bacterial Ghosts (BGs) through E-lysis has been described. An improved method for assessment of E-lysis performance in the model *Escherichia coli* was established. Bacteria become translucent during E-lysis what caused a decreased forward scatter signal of the corresponding cells. In combination with viability information obtained from staining the cells with the membrane potential-sensitive fluorescent dye *bis*-\((1,3\text{-dibutylarbituric acid})\) trimethine oxonol (DiBAC\(_4\)(3)) the method allowed to unambiguously separate viable intact cells and Bacterial Ghosts. Using a second fluorescent dye RH414 as a membrane marker it was possible to reduce non-cellular background from the data which greatly improved the quality of the results. FCM data correlated well with cell count data obtained from colony forming units (cfu) determinations for viable populations. From FCM data the rate of E-lysis for *E. coli Nissle 1917* (EcN) could be calculated to \(k = 5.6 \text{ h}^{-1}\). FCM was established as a standard parameter for evaluation of E-lysis in 20.0 l batch processes for EcN (pGLysivb) BG production.
5.1 Introduction

The Bacterial Ghost (BG) platform system is a promising new technology for vaccines and drug delivery vehicles [18, 24, 32]. Derived from Gram-negative bacteria BGs are empty cell envelopes with sustained cellular morphology. BGs are produced from viable bacteria by controlled expression of the cloned bacteriophage φX174 lysis gene E from suitable plasmids [11]. The encoded protein E is a short (91 aa), non-enzymatic [6, 23], membrane associated protein [1] that in dividing cells targets cell division sites where it oligomerizes [2] and induces fusion of the inner and outer membrane [35]. This eventually results in the formation of a distinct trans-membrane tunnel structure sealing the periplasmic space [34, 35]. Driven by the osmotic pressure difference between the cytoplasm and the surrounding medium the cytoplasm with all its constituents is expelled to the medium leaving behind an empty BG. This process is called E-lysis and has been reviewed in several papers [18, 19, 21, 24–26, 28, 32].

Depending on the designated vaccine application the BG morphology can be augmented with foreign target antigens (both protein and DNA) prior to E-lysis. Target antigens may be incorporated into the inner or outer membrane via fusion with specific membrane anchors [13, 31] or exported to the periplasmic space using adequate signal sequences so they are retained with the BG envelope during E-lysis [35]. In addition BGS have shown to be potent carriers for targeted drug delivery [18, 26].

The concept of fluorescence-based flow cytometry (FCM) for quantification and examination of single cells goes back to the 1960’s when the first such flow cytometer was patented and commercialized by PARTEC GMBH (Münster, Germany). The technique has been described [27, 30]. Assays for detection of numerous structural and functional cell characteristics have been described; such as cell viability [12], membrane integrity [4, 15], DNA and protein contents [3] and many more. Combining different scatter and fluorescence signals FCM also allows for multiparameter assessment of single cell properties depending on the available assays [15]. One major advantage of FCM over many other methods is that it evaluates large quantities of cells (> 100,000) in a very short time after sampling.
Focusing on evaluation of cell viability in bacterial cultures FCM plays an important role. Conventional determination of viable cell counts on agar plates implies some disadvantages. Results are available only with a delay of some hours or even days and also may be somewhat incorrect in case individual cells are unable to sustain growth on solid media. Furthermore, reliable kinetics of cell death cannot be established by culture based methods since time-dependent effects may proceed on the plates leading to false results [4].

For assessment of cell viability via FCM it is a common strategy to use membrane potential-sensitive dyes. One approach is to treat suspended cells with uncharged fluorogenic substrates (e.g. fluorescin esters) that - upon uptake into the cell - are unspecifically hydrolyzed to fluorescent compounds. Retention of the highly polar, fluorescent products requires intact cell membranes identifying viable cells [4,16]. As opposed to this approach anionic oxonol dyes label cells with a reduced or defect membrane potential. The dye bis-(1,3-dibutylarbituric acid) trimethine oxonol (DiBAC4(3)) only penetrates cells lacking a membrane potential where it accumulates via association with hydrophobic sites of intracellular components (proteins, membrane compartments) [7]. FCM viability assays using DiBAC4(3) have been described [4,15] and tested for the effect of antibiotics on bacterial cultures indicating that DiBAC4(3) used in FCM is a suitable and reliable indicator for cell death [16].

Assessment of BG formation through flow cytometry has been described by Haidinger et al. [8,9]. Overexpression of green-fluorescent protein (GFP) in the cytoplasm of E. coli prior to E-lysis induction was used to tag viable cells for FCM measurements. As the cytoplasm is expelled during E-lysis BGs could be identified by a loss of fluorescence [8]. In case that no fluorescent component is available in the cytoplasm another method for BG detection was developed. The previously mentioned loss of cytoplasm during E-lysis makes BGs significantly more translucent than whole cells and this affected the scatter signals in FCM. In combination with a membrane potential-sensitive dye discrimination between live cells, whole dead cells as well as BGs was shown [9]. Haidinger et al. have generated their data with a FACScalibur flow cytometer (BECTON DICKINSON, Franklin Lakes NJ, USA) using alignment beads for quantification. They suggested the combined detection of SSC and fluorescence signals after staining with DiBAC4(3) at a concentration of 1.0 µM with no incubation time [9].

Development of an industrial production process for BGs for the use as vaccines and drug delivery vehicles is currently promoted. In order to have a reliable real-time tool for assessment of E-lysis performance during BG production the assay using the shift in scatter
and staining with fluorescent dye DiBAC$_4$(3) as suggested by Haidinger et al. was adapted for a state of the art flow cytometer introducing specific changes for improvement of data quality.

### 5.2 Materials and methods

#### 5.2.1 Flow cytometer and data analysis

All data presented in this work was generated using a CyFlow SL flow cytometer from Partec (Münster, Germany) with a 488 nm blue solid state laser. The machine features true volumetric absolute counting with a sample size of 200 µl. Three fluorescence channels are available (FL1: 527 nm bandpass filter, bandwidth 30 nm; FL2: 590 nm bandpass filter, bandwidth 50 nm; FL3: 630 nm longpass filter) alongside forward (FSC) and side scatter (SSC) detection. 1,200 µl samples were prepared in a suitable dilution so that the count rate could be adjusted between 800 and 1,100 events per second by varying the sample speed between 0.5 and 3.

Since the CyFlow SL provides true volumetric absolute counting the use of alignment beads is obsolete. Absolute counts are displayed as (ml$^{-1}$) and only have to be corrected for the applied dilution. Data was collected and analyzed with the software FloMax V 2.52 (Partec). The FSC channel was appointed as trigger for particle counting. The gain settings for the different channels were as follows. FSC: 190, SSC: 300, FL1: 500, FL2: 565, FL3: 535. Data was provided as counts per ml for all designated gates; this information was exported to MS Excel and corrected for the sample dilution for subsequent determination of total cell counts, viability and progress of BG formation by the operator.

Using FloMax data could be presented as histograms or 2D dotplots. For presentation as 3D dotplots software WinMDI V 2.9 was used. The software is available f.o.c. online: http://facs.scripps.edu/software.html (as of 05/17/2011).

#### 5.2.2 Staining protocol

Potential-sensitive dye DiBAC$_4$(3) (abs./em.: 493/516 nm, FL1) was used for the assessment of viability. Stocks of DiBAC$_4$(3) were prepared in dimethyl sulfoxide (DMSO, 0.5 mM) and stored at -20°C. Fluorescent dye RH414 was used for staining of plasma membranes yielding strong red fluorescent enhancement [17]. Stocks of RH414 (abs./em.: 532/760 nm, FL2) were prepared in DMSO (2.0 mM) and stored at -20°C. Both dyes were purchased
Chapter 5 - RT assessment of E-lysis in *E.coli* through FCM

from AnaSpec (Fremont CA, USA). Depending on the anticipated cell concentration the dyes were added at equal ratios between 0.75 and 1.5 µl per ml diluted sample.

5.2.3 Production of BGs

BGs were derived from *Escherichia coli* NM522 (EcNM522) and probiotic *Escherichia coli* strain Nissle 1917 (EcN). EcNM522 was obtained from Stratagene (La Jolla CA, USA). Native EcN carries two cryptic plasmids protecting the strain against mobile genetic elements. Removing those plasmids yields EcNΔ which was provided by Ardeypharm (Herdecke, Germany). All BGs described here were produced using the plasmid pGLysivb shown in figure 5.1 [20]. The plasmid carries a gentamicin resistance gene and the lysis gene *E* under transcriptional control of a modified, temperature inducible λpR-Δ857P/O-system [14] allowing for bacterial growth at 35°C. E-lysis was induced by a temperature up-shift to 42°C. Transformation was done by electroporation with subsequent identification of positive clones on gentamicin supplemented Agar plates. Positive clones were tested and further selected with respect to growth and E-lysis performance. Working stock of selected clones were produced as 25% (v/v) glycerol stocks and stored at -80°C.

Production of BGs is generally divided in three different stages: the growth phase (duration depending on process strategy), the E-lysis phase (90 to 120 min) and product recovery (downstream processing). The point of E-lysis induction (LI) is referred to as timpoint 0 min with the preceeding growth phase being counted in negative minutes (see fig. 5.2).

All fermentations were performed in a 30 l fermenter Techfors-S (INFORS-HT, Bottmingen, Switzerland). EcNM522 BGs were produced as batch fermentations using animal-protein free (‘vegetarian’) low salt Luria-Bertani medium LBv: 10.0 g/l soy pepton (Roth, Karlsruhe, Germany), 5.0 g/l yeast extract (Roth), 5.0 g/l NaCl. Pre-cultures were grown in 4 x 500 ml LBv. Production of EcN BGs was done as batch fermentation in minimal medium adapted from DeLisa et al. [5]: 22.00 g/l C₆H₁₂O₆ · H₂O, 13.30 g/l KH₂PO₄, 4.00 g/l (NH₄)₂HPO₄, 1.70 g/l C₆H₈O₇ (citric acid), 1.20 g/l MgSO₄ · 7 H₂O, 0.1000 g/l C₅H₆O₂ · Fe, 0.0084 g/l EDTA, 0.0130 g/l Zn(CH₃COO)₂ · 2 H₂O, 0.0025 g/l (NH₄)₂HPO₄, 0.0012 g/l CuCl₂ · 2 H₂O, 0.0030 g/l H₃BO₃, 0.0025 g/l Na₂MoO₄ · 2 H₂O, 0.0045 g/l thiamine HCl, 1.00 g/l poly-propylene glycol (PPG - Sigma, St. Louis MO, USA). Pre-cultures (3 x 500 ml) were grown in the same medium (DeLisa, no PPG) with reduced glucose concentration of 8.0 g/l. All media were prepared with de-ionized water and post-sterilization supplemented with 20 mg/l gentamicin sulfate (Roth).
5.2.4 Sampling routine and cell count analysis

Depending on the process strategy samples during bacterial growth phase were taken routinely every 30 min (batch in LBv medium) or 60 min (batch in DeLisa medium). During E-lysis phase samples were taken every 30 min. Samples were analyzed with respect to the optical density at $\lambda = 600$ nm (OD$_{600}$), colony forming units (cfu) and FCM. Determination of cfu was done using a WASP spiral plater (Don Whitley Scientific, Shipley, UK) for plating and a ProtoCOL SR 92000 colony counter (Synoptics Ltd, Cambridge, UK). All dilutions were prepared as series of $10^{-1}$ and $10^{-2}$ dilution steps in sterile 0.85% (w/v) saline solution. Cell count analyses for individual samples by cfu and flow cytometry were conducted from the same dilution series for comparability.

5.2.5 Lysis efficiency

The ratio of BGs formed and viable cells at LI is defined as the E-lysis efficiency (LE). At any time $t$ after LI it can be determined indirectly from the cfu as follows:

$$LE = \left(1 - \frac{cfu(t)}{cfu(t_o)}\right) \cdot 100\%$$

where $t_o$ is the timepoint of LI. With flow cytometry results the E-lysis efficiency can be calculated directly as the ratio of cell counts (cc) in the regional gate R3 (BGs) and total cell counts (sum of R1, R2, R3):

$$LE = \left(\frac{cc_{R3}(t)}{cc_{tot}(t)}\right) \cdot 100\%$$

Definition of gates R1-3 is provided with the results.

5.3 Results

Figure 5.2 shows the viability curve of a batch fermentation for EcN (pGLysivb) BGs. During growth phase the number of viable cells increased exponentially as indicated by both optical density and cfu reaching a maximum at the timepoint of E-lysis induction (LI). Within 30 min after LI the viability of the culture decreased significantly as E-lysis initially commenced exponentially. 90 min after E-lysis the vast majority of cells had turned into BGs.
5.3.1 Establishment of a FCM strategy

The FSC signal in FCM is used to conclude cell size while the SSC signal is a measure for cell complexity or granularity [30]. The loss of cytoplasm through E-lysis should therefore be well detectable in SSC. This assumption has been confirm by the findings of Haidinger et al. [9]. Data generated with the procedure described here suggested that the distinction between whole and E-lysed bacteria is better in FSC. FCM setup requires a trigger establishing a threshold for particles to be counted. This is commonly defined in the FSC channel and also in this procedure. Since the FSC channel was at the same time used for BG detection the threshold could not be set very high without eliminating E-lysed cells from the data. As a result background noise could not be eliminated efficiently which raised the need for a secondary filter parameter. This parameter was fluorescent dye RH414 for staining plasma membranes.

Figure 5.3 shows histograms obtain from FCM for two samples taken from a batch fermentation run with EcNM522 (pGLysivb) BGs at 30 min prior to LI ((a) to (f)) and at the end of E-lysis phase (120 min, (g) to (l)). Figures 5.3 (d) and (j) show the definition of gate RN6 for RH414-positive particles (bacteria, BGs) in the histograms of channel FL2. Even though due to its emission maximum fluorescence intensity of RH414 was higher in channel FL3 (red) gate RN6 was defined in channel FL2 (orange) where the fluorescence intensity was slightly lower but separation between cells and background was better (see fig. 5.3 (d)/(j) vs. (e)/(k)). Applying gate RN6 to all other relevant histograms and dotplots allowed for exclusion of non-cellular noise. Figures 5.3 (a) and (b) as well as (g) and (h) show histograms of FSC without and with applying gate RN6, respectively.

Regional gates RN1 and RN2 were defined in order to discriminate between whole and E-lysed cells, respectively, based on cell translucency as illustrated in figures 5.3 (b)/(h) for FSC and (c)/(i) for SSC. As gating was more conclusive in FSC translucency of BGs was picked up in this channel. Figures 5.3 (f) and (l) show histograms for channel FL1 with the definition of regional gates RN3 and RN4 aiming to discriminate cells with and without polarized membranes, respectively. For empty cell envelopes fluorescence enhancement as caused by accumulation of DiBAC$_4$(3) apparently was not as distinct as has been reported for whole cells with depolarized membranes. Clear separation between viable cells and BGs based on fluorescence (FL1) was impossible. In order to distinguish between live, whole dead and E-lysed bacteria a combination of the information obtained from channels FSC and FL1 was used. In dotplots displaying FL1 vs. FSC the following regions R1 to R3 contain:
• whole, live cells (R1): FSC↑, FL1↓
• whole, dead cells (R2): FSC↑, FL1↑
• empty BGs (R3): FSC↓, FL1↑

Since the separation in FL1 was ambiguous quadrant gating did not work for this method. As recommended by the manufacturer of the flow cytometer regions R1-3 were set manually as polygons. It should be mentioned that polygonal gates R1-3 never matched completely between different fermentation runs. For every fermentation run gates R1-3 were set so they matched dotplots of all samples of the fermentation. Gate RN6 was adapted individually for each sample to give the best possible separation of cells and background.

5.3.2 Batch fermentation of EcNM522 (pGLysivb) BGs in LBv

Figure 5.4 depicts samples from a 20.0 l batch fermentation with EcNM522 (pGLysivb) BGs in LBv medium showing both 2D and 3D dotplots of samples taken at -30 min, 30 min and 90 min (end of E-lysis phase) relative to LI. During growth phase the viable culture was represented by a nice and discrete population in region R1 (fig. 5.4 (a),(b)). After LI the population shifted away from R1; the first sample after LI (30 min, fig. 5.4 (c),(d)) showed some kind of transition state. The vast majority of the population was no longer found in R1 but also had not yet completely shifted to region R3. In order to quantify this transition state relocation of region R3 was necessary as indicated exemplarily with the region R3-a (dotted lines) in figure 5.4 (d). At the end of E-lysis phase (fig. 5.4 (e),(f)) the shift from R1 to R3 was complete. The distribution of cell envelopes within R3 was less discrete than for the viable population in R1.

5.3.3 Batch fermentation of EcN (pGLysivb) BGs in DeLisa medium

Batch fermentation EcN (pGLysivb) BGs in minimal DeLisa medium has been developed as an industrial production process. During the 270 min growth phase densities close to $1 \cdot 10^{10}$ cells per ml are reached. The 120 min E-lysis phase incorporates a complete exchange of the fermentation volume through dia-filtration partly removing the expelled cytoplasmic contents. Five complete production cycles of EcN (pGLysivb) BGs were performed in DeLisa medium and monitored by FCM. Figure 5.5 depicts 2D dotplots of samples taken during two of those production runs (B01 and B02). The polygonal gates R1, R1 and R3 were conserved between the different production runs; only minor changes were necessary to fit the collected data.
Cell count data for viable cells during growth phase of all five runs were gathered through FCM as well as by CFU determination. A plot of cell counts as obtained from both methods vs. time is shown in figure 5.6. For each sample point the mean values for both methods are displayed. For either method the results were fitted with an exponential model (trendlines shown in fig. 5.6) to show consistency and determine the growth rate $\mu_{max}$. Fitting the data gave a values of $0.760 \text{ h}^{-1}$ and $0.766 \text{ h}^{-1}$ for CFU and FCM data, respectively.

Figure 5.7 shows cell count data (OD$_{600}$, CFU and FCM) for two batch fermentations runs (B01/B02) focusing on a time frame 90 min before and after LI (-90 to 90 min). Cell counts obtained from both CFU determinations and FCM matched for each run before LI. After LI there was a drop in both optical density and the number of viable cells caused by the onset of E-lysis. The shape of the OD$_{600}$ curve formed an almost perfect isosceles triangle with the abscissa 60 min before and after LI. Cell count data showed a much more rapid, initially exponential progress of E-lysis with a later retardation where only minor decrease in viable cell counts was detected. 30 min after LI there was significant difference between the results from CFU and FCM. CFU data indicated that the exponential decline of viable cells was very fast and flattened notably after 30 min. The exponential decline in cell counts from FCM data proceeded less rapidly and extended over 60 min. For samples taken at 60 min and later the cell counts determined from both methods matched again. Fitting the exponential parts of the curves (0 to 30 min for CFU, 0 to 60 min for FCM) to an exponential model yielded the rates of E-lysis $k_i$. Those rates were almost twice as high when calculated from the CFU ($k_i = 11.62$ and $10.92 \text{ h}^{-1}$ for B01 and B02, respectively) as compared to the the rates calculated from FCM ($k_i = 5.93$ and $5.70 \text{ h}^{-1}$, B01/B02). Exploiting FCM data from all five runs the average rate of E-lysis for EcN (pGLysivb) BGs produced from batch fermentation in DeLisa medium was $k_i = 5.61 \text{ h}^{-1}$ (FCM) and $11.16 \text{ h}^{-1}$ (CFU).

Table 5.1 shows the mean values for cell yield, LE's at 60 and 120 min as well as the rate of E-lysis $k_i$ obtained from both CFU and FCM determinations.

### 5.4 Discussion

Based on the method for monitoring E-lysis in *Escherichia coli* described by Haidinger et al. [9] an improved procedure for evaluation of BG production was developed. BGs could be distinguished from viable cells by a combination two different parameters, i.e. increased translucency due to the loss of cytoplasmic contents and a loss of the membrane potential. The former was detectable directly as a diminished scatter signal while the latter
was detected using membrane potential-sensitive dye DiBAC$_4$(3) [15]. Best separation of whole cells and empty BGs based on scatter was found in the FSC signal. Introducing dye RH414 as a membrane marker greatly improved the quality of FCM data as non-cellular noise was effectively removed. It was shown that red-fluorescent dye RH414 should be picked up in channel FL2 (orange). Regional gate RN6 was defined in FL2 to identify cellular particles and applied as a filter during further analysis of FCM data. The benefit of this filter was particularly great for FSC signals as noise at the low intensity range of FSC could be eliminated without affecting detection of translucent BGs. Tagging BGs with DiBAC$_4$(3) was not as conclusive as has been described for whole dead cells [4]. This might be due to the fact that the cytoplasm was expelled and thus the availability of hydrophobic sites for accumulation of the dye in the BGs was decreased. Especially E-lysed bacteria that gained a high translucency apparently did not accumulate as much DiBAC$_4$(3) and vice versa. As a result the population of BGs and the corresponding polygonal gate R3 spread over a much larger area than the viable population located in region R1. This made quadrant gating in FSC/FL1 dotplots impossible.

Haidinger et al. reported a transition state where parts of the population were found in region R2 between 10 and 20 min after LI. This subpopulation had apparently lost membrane polarization but had not yet been subject to complete E-lysis [9]. This effect was interpreted as an intermediate state where integration of protein E into the cytoplasmic membrane caused the membrane potential to collapse [34] as a first step in the E-lysis process [9]. The first samples during the production process described here were taken 30 min after LI and this effect could not be observed as such. However, the samples taken at 30 min indicated some kind of transition state as well as the majority of bacteria was no longer located in region R1 but also had not yet settled at the final location of region R3 which corresponds with the results of Haidinger et al. Starting from the sample taken 60’ after LI the BG population was located in R3 and did not shift any more for the rest of the observation period.

Consistency between cell counts obtained from regular cfu determination and FCM data during growth phase of batch fermentations with EcN (pGLysivb) was outstanding. Also, results for LE at 60 and 120 min after LI showed good conformance. At the timepoint 30 min after LI the results for viable cells deviated between cfu and FCM suggesting very different rates of E-lysis. It has been claimed that plating on solid media under certain circumstances may be inadequate for proper determination of viable cell counts [29]. E-lysis in an individual cell becomes independent from both transcription and translation within
one minute after effective induction of gene $E$ expression [33]. The actual onset of E-lysis happens about 8 to 10 min later [33]. This is a plausible explanation why a fraction of cells that had already started to express gene $E$ but had not yet lysed still appeared as viable cells in FCM in the samples taken 30 min after LI. E-lysis in cells belonging to this fraction proceeded nonetheless which is why they subsequently did not form colonies on the plates. As results from FCM are available in less than 10 min after sampling the method is arguably more suitable than cfu determination for quantification of the dynamic progress of E-lysis. The rate of E-lysis should consequently be calculated from FCM rather than cfu data. For batch fermentations of EcN (pGLysivb) BGs in minimal medium the mean rate of E-lysis $k_1$ was 5.6 h$^{-1}$.

Halfmann et al. calculated the rate of protein E-induced lysis of *E. coli* grown in rich medium culture flasks from the drop in optical density. They reported rates of E-lysis around 1.1 h$^{-1}$ [10]. Those values essentially corresponded to the growth rate observed before LI. This relation between the growth rate and rate of E-lysis obtained from the OD was also mentioned by Lubitz et al. [22] and was further substantiated by OD$_{600}$ measurements for batch fermentation of EcN (pGlysivb) BGs. The OD$_{600}$ curve shaped an isosceles triangle with the time axis indicating similar rates for growth and E-lysis. The rates calculated from FCM data, however, revealed that the rate of E-lysis was indeed much higher.

The described FCM method yielded reliable quantitative data and results were reproducible. Introducing a fluorescence-based filter parameter for tagging bacteria significantly improved the quality of FCM data. For every sample between 100,000 and 200,000 cells were evaluated emphasizing the statistical value of FCM. Results were available immediately as opposed to cfu data that required incubation of several hours at least. With its features FCM provides a tool for real-time decision making during production of EcN (pGLysivb) BGs. Where required samples could be analyzed every 10 min to further elucidate the kinetics of the dynamic E-lysis process.
5.5 Figures and tables

Figure 5.1: Plasmid map for E-lysis plasmid pGLysivb


<table>
<thead>
<tr>
<th></th>
<th>yield (cells/ml)</th>
<th>LE (60 min)</th>
<th>LE (120 min)</th>
<th>$k_l$ (1/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cfu data</td>
<td>$7.94 \cdot 10^9 \pm 1.38 \cdot 10^9$</td>
<td>$99.766 \pm 0.048%$</td>
<td>$99.461 \pm 0.176%$</td>
<td>11.16</td>
</tr>
<tr>
<td>FCM data</td>
<td>$7.15 \cdot 10^9 \pm 1.83 \cdot 10^9$</td>
<td>$99.532 \pm 0.296%$</td>
<td>$99.613 \pm 0.232%$</td>
<td>5.61</td>
</tr>
</tbody>
</table>

Table 5.1: Comparison of data obtained from cfu and FCM determination

Mean values for important parameters of five 20.0 l batch production cycles of EcN (pGLysivb) BGs as determined from cfu and FCM data. Yield: viable cells at LI. LE: E-lysis efficiency, ratio of BGs in the population in min after LI. $k_l$: rate of E-lysis. LI: E-lysis induction by T-shift (35 to 42°C)
Chapter 5 - RT assessment of E-lysis in *E.coli* through FCM

Figure 5.2: Viability of an EcN culture during a BG production process

20.0 l batch fermentation of EcN (pGLysivb) BGs in DeLisa medium. Viability as indicated by cell counts (cfu) and optical density (OD$_{600}$). LI: E-lysis induction. (a) mid growth phase. (b) towards end of growth phase. (c) 30 min after LI. (d) 90 min after LI, towards end of E-lysis phase. LI: E-lysis induction by T-shift (35 to 42°C).
Samples taken from a 20.0 l batch fermentation with EcNNM522 (pGLysivb) BGs. Histograms (a) to (f): sample 30 min prior to LI, only viable cells. Histogram (g) to (l): sample 120 min after LI (end of E-lysis phase), > 99.9% BGs.

(d)/(j): definition of regional gate RN6 identifying cells stained with RH414 - (d)/(j) vs. (e)/(k): better separation of cells and background in FL2 vs. FL3 - (a)/(g) vs. (b)/(h): effect of noise exclusion by applying gate RN6 as a filter to histograms of FSC - (b)/(h) vs. (c)/(i): separation of whole cells (RN1) and BGs (RN2) based on translucency in FSC (good) vs. SSC (ambiguous) - (f)/(l): separation of cells with polarized (RN3) and depolarized membranes (RN4), stained with DiBAC$_4$(3), in FL1 (ambiguous).
Figure 5.4: Flow cytometry for batch fermentation of EcNM522 (pGLysivb) BGs in LBv

Samples taken from a 20.0 l batch fermentation of EcNM522 (pGLysivb) BGs in LBv. (a), (b): 3D/2D dotplots FSC vs. FL1, 30 min prior to LI. (c), (d): 3D/2D dotplots FSC vs. FL1, 30 min after LI. Note that region R3 has been relocated for quantification. (e), (f): 3D/2D dotplots FSC vs. FL1, 90 min after LI (end of E-lysis phase). LI: E-lysis induction by T-shift (35 to 42°C).
Figure 5.5: Flow cytometry for batch fermentations of EcN (pGLysivb) BGs in DeLisa medium

Samples taken from two identical 20.0 l batch fermentation runs (B01 and B02) of EcN (pGLysivb) BGs in DeLisa. (a) to (c) 2D dotplots FCS vs. FL1 for run B01. (d) to (f) 2D dotplots DCS vs. FL1 for run B02. (a), (d): sample taken at LI. (b), (e): sample taken 30 min after LI. (c), (f): sample taken 120 min after LI (end of E-lysis phase). LI: E-lysis induction by T-shift (35 to 42°C).
Cell count data from the growth phase of five 20.0 l batch fermentation runs of EcN (pGLysicb) BGs in DeLisa. Cell count data as determined by cfu and FCM incl. exponential trendlines fitting both data.

Figure 5.6: Cell count data for batch fermentations of EcN (pGLysicb) BGs in DeLisa
Figure 5.7: Rates of E-lysis for batch fermentations of EcN (pGLysivb) BGs in DeLisa

Cell count data from two identical 20.0 l batch fermentation runs (B01 and B02) of EcN (pGLysivb) BGs in DeLisa. Time window 90 min before and after LI. Cell count data as determined by cfu and FCM incl. exponential trendlines fitting both data before and after LI. OD_{600} data for B01: plotted on secondary axis (logarithmic, not shown), scale adjusted to match cell count data.
5.6 References


Process Development for Industrial Scale BG Production


High-density fermentation of

*Escherichia coli* Nissle 1917 for

Bacterial Ghost production

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Bacterial Ghosts (BGs) are empty cell envelopes derived from Gram-negative bacteria by E-lysis. E-lysis preserves the bacterial morphology providing BGs with intrinsic adjuvant and targeting properties. BGs of probiotic *Escherichia coli* strain Nissle 1917 (EcN) are promising candidate carriers of subunit vaccines, active substances and therapeutic drugs or may be administered as supplements providing an unspecific immune-boost. A batch and fed-batch production process for EcN BGs were developed aiming for high cell densities before E-lysis induction and good E-lysis efficiencies (LE). With the batch process cell densities close to $10^{10}$ cells per ml were achieved while the fed-batch approach yielded close to $10^{11}$ cells per ml. LEs in both cases were $> 99.75\%$. Fermentations were followed with flow cytometry and radio-frequency impedance measurements for real-time assessment of process performance. The BG product was collected and refined through tangential flow filtration. Final inactivation of surviving bacteria was accomplished with a minimal concentration of β-propiolactone. EcN BG products are presented as a bioburden free freeze-dried powder.
List of abbreviations


6.1 Introduction

Bacterial Ghosts (BGs) are empty cell envelopes obtained from Gram-negative bacteria. BGs are produced by controlled expression of the single cloned lysis gene E from bacteriophage φX174 [1]. The gene E product, protein E, integrates into the inner membrane where it targets membrane adhesion sites, oligomerizes and induces the formation of a transmembrane tunnel structure fusing the inner and outer membrane and sealing the periplasmic space [29,31]. The key characteristic of E-lysis is that it releases the cytoplasmic contents to the medium while the cell envelope remains structurally intact [30,31]. Consequently, a BG resembles the bacterium it is derived from but is devoid of DNA and no longer reproducible. Since the cell morphology is retained also all antigenic properties are preserved within the BG [6]. Due to their nature BGs carry intrinsic adjuvant properties and are able to target specific tissues [12,19]. This allows for BGs to be used as candidate vaccines [16,27] or as drug delivery vehicles [12,18]. Using recombinant DNA technology foreign target antigens can be incorporated into the envelope complex prior to E-lysis [20,26].

The probiotic Escherichia coli strain Nissle 1917 (EcN) is named after Prof. Alfred Nissle, MD, (Freiburg, Germany) who in 1917 isolated the bacterium from the stool of a soldier who in the field did not get affected by diarrheal diseases. It is characterized by outstanding antagonistic strength against a broad variety of enteropathogens (e.g. pathogenic E. coli, Shigella and Salmonella strains, Vibrio cholerae). Upon colonization of EcN in the gastrointestinal tract settling of pathogenic enterobacteria is efficiently prevented. This property is linked to the ability of EcN to spread and form stable biofilms in the colon as well as to produce anti-bacterial microcin M and microcin H47. At the same time EcN does not exhibit any pathogenicity factors (i.e. enterotoxins, cytotoxins, α-hemolysin, P-/S-fimbriae and other) [22,24]. The genome structure of EcN (serotype O6:K5:H1) has been analyzed [4,25] giving important knowledge about the characteristics of the strain. EcN
has a signature lipopolysaccharide (LPS) with a truncated polysaccharide chain making it serum-sensitive [5]. The shortened LPS also is the reason why EcN is immunomodulatory active but does not cause sepsis [22]. EcN carries two cryptic plasmids (pMUT1/2) with unknown function, however, the plasmids apparently protect the strain against mobile genetic elements [24]. Using the uniqueness of the plasmids a EcN-specific multiplex PCR method could be established for strain identification [2]. Viable EcN is the active ingredient in the drug Mutaflor® (ARDEYPHARM, Herdecke, Germany) which has been marketed since 1917. It is prescribed for patient suffering from chronic inflammatory or functional bowel diseases and is even used for the treatment of infants [22]. It is further applied as an immunostimulant [24].

Growing bacterial and especially *E. coli* cultures to high densities has been studied intensively since the 1970’s. A major limitation to reach elevated cell densities of *E. coli* in regular batch processes is given by the inhibitory effect of high nutrient concentrations on cell growth. This problem was overcome with the development of fed-batch or dialysis techniques. By this means cell densities well above 100 g/l dry cell weight could be achieved [11, 17, 23]. Limitations to high-density processes arise from limited solubility of substrates (both solid and gaseous), accumulation of inhibitory levels of side-products (e.g. acetate), foaming or stress response to heterologous protein expression [21, 23].

Combining the features of probiotic EcN with the concept of BGs highly promising candidate vaccines and drug carrier systems can be developed. Due to its properties EcN is the ideal carrier system for active substances or subunit vaccines. The BG envelope resembles EcN and thus does not exhibit any toxic or pathogenic properties. Moreover the EcN morphology is expected to maintain its immunostimulatory features and thus act as an adjuvant. Low density batch fermentations of BGs in complex medium yields no more than $10^9$ cells per ml [13]. In order to increase the yield of the production process for EcN BGs batch and fed-batch processes in minimal medium have been developed.

### 6.2 Materials and Methods

#### 6.2.1 Strain and plasmid

In order to be genetically modified the cryptic plasmids pMUT1/2 have to be removed from EcN leading to the modified form EcNΔ. EcNΔ stocks were obtained from ARDEYPHARM (Herdecke, Germany) and transformed with lysis plasmid pGLysivb [14]. pGLysivb is shown in figure 6.1, it carries the E-lysis cassette under control of the modified, temperature-
inducible $\lambda_p$-d857 promoter/operator-system as well as a gentamicin resistance gene. The modified P/O-system allows for tight control of gene $E$ expression at the growth temperature of 35°C; a rapid T-shift to 42°C induces gene expression [7]. Transformation was performed by electroporation and verified on agar plates containing gentamicin. Preliminary testings for growth and expression of lysis gene $E$ identified the most promising clone for working stock production. Working stocks were produced as 25% (v/v) glycerol stocks (1.8 ml) and stored at -80°C.

6.2.2 Culture media

Fermentations were performed in defined medium as suggested by DeLisa et al. [3]. The batch medium provides 20 g/l of glucose as primary carbon source. The composition is:

$$
22.00 \text{ g/l } C_6H_{12}O_6 \cdot H_2O, 13.30 \text{ g/l } KH_2PO_4, 4.00 \text{ g/l } (NH_4)_2HPO_4, 1.70 \text{ g/l } C_6H_{12}O_6 \cdot H_2O \text{ (citric acid)}, 1.20 \text{ g/l } MgSO_4 \cdot 7 H_2O, 0.1000 \text{ g/l } C_6H_{12}O_6 \cdot Fe, 0.0084 \text{ g/l } EDTA, 0.0130 \text{ g/l } Zn(CH_3COO)_2 \cdot 2 H_2O, 0.0025 \text{ g/l } CoCl_2 \cdot 6 H_2O, 0.0150 \text{ g/l } MnCl_2 \cdot 4 H_2O, 0.0012 \text{ g/l } CuCl_2 \cdot 2 H_2O, 0.0030 \text{ g/l } H_3BO_3, 0.0025 \text{ g/l } Na_2MoO_4 \cdot 2 H_2O, 0.0045 \text{ g/l } \text{thiamine HCl}, 1.00 \text{ g/l } \text{poly-propylene glycol (PPG - SIGMA, St. Louis MO, USA). Pre-cultures (2 x 250 ml) were grown in the same medium (DeLisa, no PPG) with reduced glucose concentration of 8.0 g/l, the pH was adjusted to 7.20 prior to sterilization using 5 M KOH.}
$$

The feed solution provides a glucose concentration of 150 g/l. The composition is: 165.00 g/l $C_6H_{12}O_6 \cdot H_2O, 7.50$ g/l $MgSO_4 \cdot 7 H_2O, 0.01500$ g/l $C_6H_{12}O_6 \cdot Fe, 0.00488$ g/l EDTA, 0.00600 g/l $Zn(CH_3COO)_2 \cdot 2 H_2O, 0.00150$ g/l $CoCl_2 \cdot 6 H_2O, 0.00900$ g/l $MnCl_2 \cdot 4 H_2O, 0.00072$ g/l $CuCl_2 \cdot 2 H_2O, 0.00180$ g/l $H_3BO_3, 0.00150$ g/l $Na_2MoO_4 \cdot 2 H_2O, 5.00$ g/l PPG. All media were prepared with de-ionized water (dH$_2$O). The sterilized media were supplemented with gentamicin stock (10 g/l gentamicin sulfate, Roth, Karlsruhe, Germany) to a final concentration of 20 mg/l.

6.2.3 Fermentation

All fermentations were performed in a 30.0 l Techfors-s fermenter (INFORS-HT, Bottmingen, Switzerland) standardly equipped with temperature, pH, aeration and stirring control. For the fed-batch process head-pressure control and radio-frequency impedance (RFI) measurements were available in addition. Batch fermentation was monitored and controlled with the software IRIS V5 (INFORS-HT); fed-batch fermentation was managed with a central process management system (PMS) Lucullus V 3.1.0 (BIOSPECTRA, Schlieren, Switzerland).
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**Batch process**

The batch process was conducted in a working volume of 20.0 l at growth conditions of 35°C and pH 7.20. Initial aeration parameters for the fermenter were 1,000 rpm stirring and 1.0 vvm compressed air. Pre-cultures (3 x 500 ml) were grown at 35°C overnight. The process was inoculated to an OD$_{600}$ of approx. 0.3 to 0.5. Growth phase proceeded for 270 min to an OD$_{600}$ of about 15. Whenever the dissolved oxygen concentration ($dO_2$) dropped below 40% saturation the aeration rate was increased by increments of 0.5 vvm (max.: 2.0 vvm). E-lysis was induced by a T-shift to 42°C, the point of lysis induction (LI) is referred to as timepoint 0 min. The ensuing lysis phase lasted 120 min. 15 min after LI a dia-filtration system (DF) was activated. Over the course of the E-lysis phase the culture broth volume (20.0 l) was exchanged once with deH$_2$O. Downstream processing (DSP) commenced at the end of E-lysis phase with concentration of the culture broth by tangential flow filtration (TFF) to a minimum volume (< 1.0 l). The concentrated suspension was flushed from the vessel/DF system with sterile deH$_2$O to a stirred 2.0 l GSL80 Schott flask and refilled to a total volume of 2.0 l. The concentrate was immediately treated with $\beta$-propiolactone (BPL). After BPL-treatment the concentrate was further washed in a second DF-run the volume of the concentrate (2.0 l) was exchanged five times with 10.0 l sterile deH$_2$O. The washed BG suspension represents the final state of the product before bottling.

**Fed-batch process**

Fed-batch fermentation started with a 7.0 l batch fermentation ($T = 35°C$, pH 7.20, PPG reduced to 0.3 g/l) with stirring and aeration rates of 1,200 rpm and 20.0 l/min, respectively. In addition the head-pressure in the fermenter was regulated at 0.8 bar. Pre-cultures were started 12 h before inoculation of the fermenter which aimed for an initial OD$_{600}$ < 0.1 since the batch was to proceed overnight. Once the carbon-source was depleted as indicated by the $dO_2$ the feed stream was activated with the desired growth rate $\mu_f$. Feeding was actuated through the built-in peristaltic feed pump at the Techfors-S. The feed-rate set-point was calculated for an exponential feed profile by the PMS. The actual pump rate was controlled via feed-back control using the weight of the feed container as measured variable (balance: EA35EDF-I, SARTORIUS, Göttingen, Germany). During feed phase an additional flow of pure oxygen was provided to maintain a sufficient $dO_2$. At the appropriate timepoint E-lysis was induced by a T-shift to 42°C. The timepoint of LI is denoted as 0 min. The feed stream was maintained and gradually reduced during E-lysis phase.
6.2.4 Sampling and analysis

20 ml samples were taken from the process every 60 min during growth phase and every 30 min during E-lysis phase. The samples were analyzed with respect to optical density at $\lambda = 600$ nm ($OD_{600}$) and colony forming units (cfu) using a WASP spiral plater (DON WHITLEY SCIENTIFIC, Shipley, UK) for plating and a ProtoCOL SR 92000 colony counter (SYNOPTICS LTD, Cambridge, UK). DNA concentration in the culture broth supernatant was determined by simple OD-measurements at $\lambda = 260/280$ nm in an Infinite 200 Microwell Reader (TECAN, Männedorf, Switzerland) with UV-transparent 96 well plates. The residual glucose concentration in the culture broth supernatant was determined photometrically in the Infinite 200 MR using the GluePAP assay GL3981 (ROCHE DIAGNOSTICS, Basel, Switzerland). During fed-batch fermentation the same assay was used automated in a CuBiAn XC Photometric Biochemistry Analyzer (ROCHE DIAGNOSTICS, Basel, Switzerland) together with the Enzytec fluid Acetic Acid assay E5226 (r-biopharm, Darmstadt, Germany).

6.2.5 Flow cytometry

Cell counts and viability were further analyzed via flow cytometry (FCM) with a CyFlow SL flow cytometer (PARTEC, Münster, Germany) featuring true volumetric absolute counting (sample size: 200 µl). Samples were diluted appropriately and stained with 1.5 µl/ml fluorescent dyes: RH414 (cell membrane marker [9], 2 mM, DMSO) and bis-(1,3-dibutylarbituric acid) trimethine oxonol (DiBAC$_4$(3), assessment of viability [8], 0.5 mM, DMSO), each. Both dyes did not require incubation. Dyes were purchased from ANASPEC (Fremont CA, USA).

6.2.6 Lysis efficiency

The ratio of BGs formed and total particles is defined as the E-lysis efficiency (LE). At any time after LI it can be determined indirectly from the cfu:

$$ LE = \left(1 - \frac{cfu(t)}{cfu(t_0)}\right) \cdot 100\% $$

where $t_0$ is the timepoint of LI. With flow cytometry results LE can be calculated directly form the cell counts (cc) in the different regions:

$$ LE = \left(\frac{cc_{R3}(t)}{cc_{tot}(t)}\right) \cdot 100\% $$
6.2.7 RFI measurements

Radio-frequency impedance (RFI) measurement was done using a Biomass monitor 220 (Aber Instruments Ltd., Aberystwyth, UK) with an annular probe. According to the manufacturers suggestion measurements with E. coli were performed at 1 MHz.

6.2.8 Tangential flow filtration

Tangential flow filtration (TFF) was performed using a CFP-2-E-8A hollow fiber module (GE Healthcare, Chalfont St. Giles, UK) with a 0.2 µm cut-off. For all TFF applications the feed was recirculated by a Flowmaster FMT 300 peristaltic pump (ISMTEC, Glattbruck, Switzerland). For DF during E-lysis phase and concentration the BG suspension was circulated form the Techfors-S fermenter. The pressure loss \( \Delta p \) in the module was measured with a digital manometer GDH 13 AN (Greisinger, Regenstauf, Germany). The culture broth was circulated with a feed rate of 9.0 l/min and the pressure loss in the module was regulated to 400 mbar by tightening a hose clamp downstream of the TFF module. During DF the liquid level in the fermenter was replenished with a stream of sterile dH\(_2\)O delivered by a MCP Standard peristaltic pump (ISMTEC) compensating the permeate flux passing the TFF-membrane. All liquid lines were ID 16 mm Rotilabo (Roth) tubes linked with valved in-line couplings HFC/PMC series (CPC, St. Paul MN, USA). For concentration of the culture broth the fresh water stream was stopped and the \( \Delta p \) increased to 600 mbar. The second DF run (washing with 10.0 l dH\(_2\)O) was done in the 2.0 l GSR 80 Schott flask with a pump rate of 6.0 l/min and an enforced \( \Delta p \) of 800 mbar.

6.2.9 BPL-treatment

\( \beta \)-propiolactone (BPL, 98.5% pharmaceutical grade, Ferak, Berlin, Germany) was used for final inactivation of surviving bacteria in the concentrate. For the batch process a total of 0.5\%\(_{v/v}\) (\( v_f \): total fermentation volume) was sufficient (10 ml BPL). The total amount of BPL was administered in two equal dozes of 5.0 ml, each, with a delay of 30 min. Application of BPL was at 42\(^\circ\)C for a total of 60 min. Plating of undiluted concentrate (3 x 400 µl) as well as 48 h enrichment cultures (200/400 µl, concentrate in 5 ml sterile LB medium: 10.0 g/l soy peptone (Roth), 5.0 g/l yeast extract (Roth), 5.0 g/l NaCl, incubated at 35\(^\circ\)C) were done to confirm successful inactivation. The lyophilized product was also tested for sterility. 2 ml of re-suspended BGs (10 mg/ml) were prepared with sterile LB medium. 2 * 200 µl of the suspension were prepared with Koch’s plating method in 20
ml sterile Plate Count Agar (ROTH), each. 100 and 200 µl of the undiluted suspension were plated on agar plates. 100/200 µl of the suspension were incubated in 5 ml sterile LB, each, as enrichment cultures at 35°C for 24 h. 100/200 µl of the enrichment were plated. All plates were incubated at 35°C for 24 h. Total sterility of the product was verified by all tests being negative for viable cells.

6.3 Results

E-lysis has been reported to be dependent on the physiological state of the culture and is only functional in exponentially growing cells [15,28]. Therefore, during batch fermentation E-lysis should be induced before the limiting substrate is depleted. During fed-batch fermentation the feed should be maintained into E-lysis phase.

6.3.1 Batch fermentation of EcN (pGLysivb) BGs

A total of nine complete batch production cycles for EcN (pGLysivb) BGs in DeLisa medium were performed (B03-11). The established timeline for such production cycle is given in figure 6.2 (a). Inoculation of the fermenter was done with 1.5 l healthy pre-culture (OD$_{600}$ ≈ 4.0) resulting an initial OD$_{600}$ between 0.3 and 0.4. The growth phase extended over 270 min until about 60% of the glucose was consumed (residual glucose concentration: about 8 g/l). At the end of growth phase OD$_{600}$ was approximately 15 while cfu-yields ranging from 5.85 to 9.90 · 10$^9$ cells per ml were reached with an average of 7.35 · 10$^9$ ± 1.23 · 10$^9$ cells per ml. The mean maximal growth rate for EcN (pGLysivb) in DeLisa medium was calculated from cfu data: $\mu_{max} = 0.760$ h$^{-1}$. E-lysis was induced by a T-shift from 35 to 42°C at timepoint 0 min resulting in an immediate drop in viability. Figure 6.5 shows the viability curve over the course of a batch fermentation (OD$_{600}$, cfu, viable cells determined from FCM). All fermentations reached a lysis efficiency (LE) as determined from the cfu of at least 99.7% within 60 min after LI with an average of 99.782±0.051%.

Figure 6.6 illustrates the course of relevant process parameters over the batch fermentation process (temperature, dO$_2$, residual glucose). The initial aeration rate of 1.0 vvm was increased to 1.5 and 2.0 vvm when the dO$_2$ dropped below 40% saturation. The T-shift to 42°C at LI was rapid and caused a signature reaction in the dissolved oxygen curve as the dO$_2$ dropped to < 5% saturation within 3 to 5 min after LI, the curve shape then showed a bump back to about 35% at approximately 12 min and immediately fell back to below 5%. Between 27 to 29 min after LI the onset of E-lysis became evident as the dO$_2$
curve started to converge towards 100% saturation. As soon as this recovery commenced the aeration rate was reduced back to 1.0 vvm. Similar \( dO_2 \) curve shapes were observed in all batch fermentation runs (fig. 6.7). The recovery was very rapid and followed an exponential progress. The recorded data was fitted to a suitable model:

\[
dO_2 = dO_{2,\text{max}} (1 - \exp(\delta t))
\]  

\( dO_{2,\text{max}} \) was usually between 97 and 99% saturation while the mean value for parameter \( \delta \) was \( 0.362 \pm 0.026 \text{ min}^{-1} \).

Initially, two fermentation runs (B01/02) were performed without DF during E-lysis phase. After E-lysis onset the culture broth became very viscous which caused foaming and also hampered handling the samples (i.e. pipetting). In runs B03-11 the DF system was activated 15 min after LI. Over the course of E-lysis phase the culture broth volume was exchange once with 20.0 l \( \text{dH}_2\text{O} \). Figure 6.3 shows a simple flow diagram of the DF system as applied during E-lysis phase. The permeate flux \( F_{\text{perm}} \) was not constant with time and had to be monitored thoroughly to maintain a constant volume in the fermenter by adjusting \( F_{\text{DF}} \). DF was sufficient to keep the culture broth viscosity down thereby reducing foaming issues. Handling the samples was normal. The purging effect using dia-filtration in E-lysis phase was demonstrated by detection of free DNA in the culture broth supernatant (see fig. 6.8). At the end of E-lysis phase the culture broth was concentrated to a minimum volume by TFF, transferred to a stirred glass vessel and filled up to 2.0 l with \( \text{dH}_2\text{O} \). The concentrate was treated with 2 x 5.0 ml BPL administered at 30 min intervals. The BPL-treated concentrate was tested for sterility, no viable cells were detected in any sample. In a second DF-run the 2.0 l concentrate volume was washed with 10.0 l \( \text{dH}_2\text{O} \). The purging effect was again determined by measuring the DNA in the supernatant (see fig. 6.8). The washed and inactivated product suspension was spread to equal 40 ml aliquots and lyophilized. The average yield of lyophilized BGs from a 20.0 l batch fermentation was 73.12 g. The freeze-dried product was tested for sterility.

### 6.3.2 Fed-batch fermentation of EcN (pGLysivb) BGs

The suggested timeline for a fed-batch production cycle of EcN (pGLysivb) BGs is given in figure 6.2 (b). Inoculation of the preceding batch phase \( (V_0 = 7.0 \text{ l}, S_0 = 20.0 \text{ g/l glucose}) \) was done aiming for an initial \( \text{OD}_{600} \) of approximately 0.1 to extend the batch phase overnight. With a healthy pre-culture \( \text{OD}_{600} \approx 3 \) the batch phase continued for about 8 h yielding an average 7.51 g/l dry biomass (DBM) corresponding to cell counts
(cfu) of $8.53 \cdot 10^9$ cells/ml. The end of batch phase was indicated by an abrupt up-shift of the dO$_2$ to 100% saturation. The feed rate $Q$ during subsequent feed phase was increased exponentially with a pre-defined growth rate $\mu_f$. From a biomass balance for a stirred tank reactor (STR):

$$\frac{d(XV)}{dt} = QX_{in} + \mu_f XV - k_d XV$$  \hspace{1cm} (6.2)

Assuming a sterile feed $X_{in} = 0$ and neglectable cell death $k_d \ll \mu_f$:

$$\frac{dX}{dt} V + \frac{dV}{dt} X = \mu_f X$$  \hspace{1cm} (6.3)

Integration gives:

$$\frac{X}{X_1} = \frac{V}{V_1} \exp(\mu_f t)$$  \hspace{1cm} (6.4)

The suffix 1 describes the initial state at the beginning of feed phase. From a substrate balance for an STR:

$$\frac{d(SV)}{dt} = QS_{in} - q_s XV$$  \hspace{1cm} (6.5)

For growth rates $\mu_f < \mu_{max}$ substrate cannot accumulate and the left hand side of equation 6.5 is zero. The specific substrate uptake rate is defined by:

$$q_s X = \frac{1}{Y_{X/S}} \frac{dX}{dt} = \frac{1}{Y_{X/S}} \mu_f X$$  \hspace{1cm} (6.6)

Combining equations 6.5 and 6.6 leaves:

$$Q = \frac{\mu_f XV}{Y_{X/S} S_{in}}$$  \hspace{1cm} (6.7)

The initial state is known: $Q_1 = f (X_1, V_1)$. Combining equations 6.4 and 6.7:

$$Q = Q_1 \exp(\mu_f t)$$  \hspace{1cm} (6.8)

At the end of batch phase the biomass $X_1$ was estimated from the OD$_{600}$ by linear correlation from experimental results for EcN (pGLysivb): $X_1 = 0.306 \cdot$ OD$_{600} - 1.671$. The yield of biomass from substrate was also determined experimentally for EcN (pGLysivb): $Y_{X/S} = 0.44 \text{ g/g}$. The initial feed rate was calculated and implemented through the PMS together with the desired growth rate $\mu_f = 0.7 \text{ h}^{-1}$. The total feed volume was 9.0 l. At the end of feed phase average cell densities of $7.06 \cdot 10^{10} \pm 3.40 \cdot 10^{10} \text{ cells per ml}$ were reached (DBM 30 to 35 g/l).
E-lysis was induced by a T-shift to 42°C. The exponential feed profile was maintained for 5 min after LI. Afterwards the following decelerated feed profile was applied (in %, based on the last value of the exponential feed rate): 15 min at 100%, 20 min at 50%, 20 min at 25%, 30 min at 10%, 30 min at 5%. DF was not applied during E-lysis phase in the fed-batch runs but the anti-foam agent (5.0 g/l PPG) was sufficient to control foaming. Handling samples taken after LI was difficult due to increased culture both viscosity. Three runs (FB 12-14) were performed with the decelerated feeding strategy as described above. Figure 6.9 shows the viability parameters OD600 and cfu over the course of a fed-batch production cycle. The drop in cfu after LI was less rapid than observed for batch fermentations and extended over the whole 120 min of E-lysis phase. At the end of E-lysis phase (120 min) a mean LE of 99.776 ± 0.133% was accomplished. Downstream processing was not done for the fed-batch process.

6.3.3 Viability assessment by capacitance measurements during EcN (pGLysivb) BGs production

Initial fed-batch runs (FB01-11) were performed with different feeding strategies after LI. In those runs bacterial growth and E-lysis were followed by capacitance, or radio-frequency impedance (RFI), measurements. Viable bacteria in an electric field act like tiny capacitors since their membranes are able to separate charges. The very small capacitance of single cells in an electric field adds up so the measured overall capacitance in a suspension is proportional to the concentration of viable cells in the suspension. Figure 6.4 depicts the measuring principle of RFI, as adapted from Kaiser et al. [10]. E-lysis goes along with the collapse of membrane potential [30] and thus BGs were not detectable by RFI measurements. Figure 6.10 shows RFI measurements during three different fed-batch runs (FB07/08/10) with different feeding strategies after LI. In run FB07 the feed rate was kept at 100% for 30 min after LI and then reduced to 10% for another 30 min. In run FB08 the feed rate was halted at 100% for 50 min, both runs were stopped 60 min after LI. In run FB10 a decelerated feed profile over 120 min was implemented (5 min exponential, 30 min at 100%, 25 min at 50%, 60 min at 10%). The capacitance signal in all three runs stopped increasing immediately after LI and started to drop rapidly about 18 to 20 min after LI. In run FB07 the decline stopped shortly after the feed rate was reduced (30 min). In run FB08 the signal kept decreasing until the feed was stopped (50 min). In run FB10 with continued decelerated feeding the RFI signal kept dropping over the 120 min E-lysis phase. At the end of E-lysis phase the signal had declined to approximately 75% of the level at the start.
of feed phase. RFI data (capacitance $C$) could be correlated to DBM data: $\text{DBM (g/l)} = 2.258 \cdot C \text{ (pF/cm)} + 2.837$.

6.3.4 Assessment of E-lysis performance in EcN (pGLysivb) through flow cytometry

Both batch and fed-batch processes were followed through FCM using a double-stain fluorescence assay. Fluorescent dye RH414 was used as an unspecific marker for cell membranes. It was picked up in fluorescence channel 2 (FL2, orange) and used to define a gate for cellular particles which allowed to exclude non-cellular noise from the data. Membrane potentialsensitive dye DiBAC$_4$(3) was picked up in fluorescence channel 1 (FL1, green). BGs were much more translucent than viable cells and thus caused a diminished forward-scatter signal (FSC). DiBAC$_4$(3) accumulated in cells with disrupted membranes therefore staining non-living cells only. Combining information about viability obtained from channel FL1 and the translucency signal from FSC in a 2D-dotplot (FL1 vs. FSC) was used to define the following three polygonal regions (R#):

- **R1** - whole, live cells: FSC↑, FL1↓
- **R2** - whole, dead cells: FSC↑, FL1↑
- **R3** - empty BGs: FSC↓, FL1↑

For batch fermentations of EcN (pGLysivb) BGs the FCM data correlated very well with the results from cfu determinations (fig. 6.5). The calculated maximal growth rate was $\mu_{\text{max}} = 0.766 \text{ h}^{-1}$. Recurring difference between cfu and FCM data was only found 30 min after LI where the number of viable cells as detected by FCM was almost twice as high as those determined by cfu. From FCM data the rate of E-lysis in batch fermentation of EcN (pGLysivb) BGs was $k_l = 5.6 \text{ h}^{-1}$. For fed-batch fermentations correlation between cfu and FCM data could not be shown. Figure 6.11 shows a set of three 2D-dotplots, each, for a batch and fed-batch fermentation run. The dotplots depict the respective cultures before LI, 30 and 120 min after LI. In both cases the 2D-dotplots representing the end of E-lysis phase indicate successful E-lysis as the populations have completely shifted from R1 to R3.

6.4 Discussion

With the presented batch process for EcN (pGLysivb) BGs in minimal medium cell yields of almost $10^{10}$ cells per ml were reached, that corresponded to an increase of roughly one order of magnitude as compared to batch fermentations in complex medium. The process
was shown to be stable and reproducible over a total of nine runs. E-lysis efficiencies > 99.7% were achieved within 60 min after LI. Cell counts during growth phase as well as LEs at 60 and 120 min were confirmed between cfu and FCM data. The difference in cell counts obtained from both methods for samples taken 30 min after LI was attributed to the fact that cfu determination required incubation (overnight) while FCM results were available within minutes. E-lysis is a dynamic process extending over a certain timeframe [29]. This should be the reason why a fraction of cells still appeared as viable cells in FCM immediately after sampling but were later unable to form colonies on agar plates. Consequenly, the rate of E-lysis for batch fermentations with EcN (pGLysivb) should be calculated from FCM data. Furthermore, FCM provides good visualization of the E-lysis process as is allows to unambiguously distinguish between populations of live cells, whole dead cells and BGs in 2D-dotplots.

The occurrence of E-lysis in the batch process was clearly indicated by the reaction of the dissolved oxygen concentration $dO_2$ in the culture broth. The $dO_2$ that had dropped to < 5% saturation after LI started to quickly recover towards 100% saturation in an exponential manner around 28 min after LI. For other approaches (different strains/media) significantly lower recovery curves were observed (i.e. $\delta \leq 0.05 \text{ min}^{-1}$). The parameter $\delta$ does not seem to be directly linked to E-lysis kinetics as it is much higher than the rate of E-lysis $k_l$. Also, the $dO_2$-shift seems somewhat delayed with respect to the anticipated onset of E-lysis (approx. 20 min after LI). Due to the high conformity of the curve shape between different runs the $dO_2$ is still a good qualitative parameter to rate E-lysis performance.

Integrating DF into E-lysis phase was beneficial as it helped to remove cytoplasmic components from the culture broth that were expelled as a result of E-lysis. This held the culture broth viscosity down and also was expected to maintain the osmotic pressure difference between cytoplasm and culture medium as a major driving force for E-lysis [32]. The DNA concentration in the culture broth superntant was used as an example for the purging effect of DF. Removing DNA by DF before concentration and BPL-treatment additionally helped to minimize the amount of BPL required for final inactivation of surviving bacteria in the BG product [14]. Harvesting the BG product by TFF proved to be safe with respect to cross contamination as the system was operated in closed sterile loops. TFF also avoided pelletization of the BG product. Pelletization (e.g. during harvesting in a separator) in some cases led to irreversible agglomeration of BG particles before lyophilization. The freeze-dried product as described here can be re-suspended easily by adding water or buffer and shaking up the suspension.
Introducing a fed-batch process for production of EcN (pGLysivb) BGs yielded cell densities that were one order of magnitude higher than those obtained with batch fermentation in DeLisa medium. With respect to batch fermentations in complex medium the yield could be increased by almost a factor 100 without losing E-lysis efficiency. As E-lysis is best in exponentially growing cells the growth rate during feed phase $\mu_f = 0.7 \text{ h}^{-1}$ was chosen close to the maximal growth rate of EcN (pGLysivb) which was determined as $\mu_{\text{max}} = 0.76 \text{ h}^{-1}$. It was further anticipated that the feed must be maintained into E-lysis phase. This assumption was confirmed by RFI measurements since for fed-batch runs where within 60 min after LI the feed was suspended or stopped E-lysis was insufficient (LEs < 90%). As RFI data suggested that E-lysis proceeded somewhat exponential it was concluded to gradually decelerate the feed rate during E-lysis phase in a pseudo-exponential manner. Three runs performed with such decelerated feed profile after LI resulted in satisfying values of almost 99.8% for LE. RFI measurements gave good qualitative results for both growth and E-lysis phase. For growth phase the measured capacitance was correlated with the DBM, however, $R^2$ was only 0.886. Thus, reliably judging the magnitude of E-lysis from RFI data was not possible. Plotting the RFI signal vs. time still gave very good indication about the timepoint of onset and the progress of E-lysis. TFF was not performed for the fed-batch process, yet it was suggested that downstream processing as described for the batch process is applicable to the fed-process.

With the described batch and fed-batch strategies the yields of BG production could be increased by a factor 10 and 100, respectively, with good LE. TFF was sucessfully integrated in the downstream procedure resulting in improved product quality and reducing the amount of BPL required for final inactivant of the product before lyophilization. Introducing FCM and RFI as real-time parameters for assessment of process performance gave convincing results for both bacterial growth and E-lysis.

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6.5 Figures and tables

Figure 6.1: Plasmid map for E-lysis plasmid pGLysivb


Figure 6.2: Process timelines for batch and fed-batch production process of EcN BGs

(a) Established timeline for batch process including DSP (b) Established timeline for fed-batch process, subsequent DSP according to batch process. Timeframes for pre-culture and batch phase during fed-batch process may vary. Killing with BPL: two doses of 5.0 ml BPL at 30 min intervals.
Figure 6.3: *Flow diagram for dia-filtration during batch production of EcN (pGLysivb) BGs* DF as applied during E-lysis phase in the batch production process of EcN (pGLysivb) BGs. $F_{\text{ret}}$: retentate flux, recirculated. $F_{\text{perm}}$: permeate flux passing the TFF membrane. $F_{\text{DF}}$: fresh water flux ($dH_2O$).

Figure 6.4: *Principle of radio-frequency impedance measurements* Annular RFI probe immersed in a cell suspension. Cells with intact membranes (green) act as tiny capacitors in the electric field. Cells with defect or 'leaky' membranes (such as BGs, grey) are invisible to the system. Picture adapted from Kaiser et al. [10]
Figure 6.5: Viability curve for the batch production process of EcN (pGLysivb) BGs

Viability parameters OD$(_{600}$), cfu and viable cell counts obtained from FCM for a batch fermentation run of EcN (pGLysivb) BGs in DeLisa medium. LI: E-lysis induction (T-shift 35 to 42°C).
Figure 6.6: Process graph for the batch production process of EcN (pGLysivb) BGs

Relevant process parameters: oxygen (dO$_2$), temperature and glucose concentration in the culture broth supernatant. Normalization of parameters: dO$_2$ vs. 100% saturation, temperature vs. 50°C, glucose concentration vs. 20.0 g/l. air: 0.5vvm increased air flow. LI: E-lysis induction (T-shift 35 to 42°C).
Figure 6.7: Dissolved oxygen curves for different batch production cycles of EcN (pGLysieb) BGs

Re-occurring signature dO2 curve for different batch fermentations runs: (a) B08, (b) B09, (c) B10, (d) B11. Experimental data (blue) was fitted to an exponential model (green):

\[ dO_2 = dO_{2,\text{max}} (1 - \exp(\delta t)) \]
Figure 6.8: Dia-filtration during batch production of EcN (pGLysivb) BGs
 DF applied during batch production of EcN (pGLysivb) BGs. (a): DF during E-lysis phase, single exchange of 20.0 l culture broth volume. (b): DF in downstream processing, five-fold exchange of 2.0 l concentrate volume. LI: E-lysis induction (T-shift 35 to 42°C).
Figure 6.9: Viability curve for the fed-batch production process of EcN (pGLysivb) BGs

Viability parameters OD\(_{600}\) and cfu for a fed-batch fermentation run of EcN (pGLysivb) BGs in DeLisa medium. Feed start: begin of exponential feed with \(\mu_f = 0.7 \text{ h}^{-1}\). LI: E-lysis induction (T-shift 35 to 42\(^\circ\)C).
Figure 6.10: Viable biomass during fed-batch fermentation of EcN (pGLysivb) BGs as determined by radio-frequency impedance measurements. RFI measurements recorded during fed-batch fermentation runs of EcN (pGLysivb) BGs in DeLisa medium. FB07: feed ended 30 min after LI (A). FB08: feed ended 50 min after LI (B). FB10: continued feeding during 120 min E-lysis phase. LI: E-lysis induction (T-shift 35 to 42°C).
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## Glossary

### Latin symbols

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<th>Definition</th>
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<tr>
<td>$c$</td>
<td>g/l, mol/l</td>
</tr>
<tr>
<td>$C_m$</td>
<td>$\mu$F/cm$^2$</td>
</tr>
<tr>
<td>$d$</td>
<td>mm</td>
</tr>
<tr>
<td>$d_p$</td>
<td>$\mu$m</td>
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<tr>
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<td>mm</td>
</tr>
<tr>
<td>$E$</td>
<td>-</td>
</tr>
<tr>
<td>$f_c$</td>
<td>MHz</td>
</tr>
<tr>
<td>$F_{DF}$</td>
<td>ml/min</td>
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<tr>
<td>$F_{perm}$</td>
<td>ml/min</td>
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<tr>
<td>$H$</td>
<td>mm</td>
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<td>h$^{-1}$</td>
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<td>$p$</td>
<td>mbar</td>
</tr>
<tr>
<td>$P$</td>
<td>-</td>
</tr>
<tr>
<td>$q_s$</td>
<td>g$_x$/g$_x$h</td>
</tr>
<tr>
<td>$Q$</td>
<td>ml/min</td>
</tr>
<tr>
<td>$Q_{in}$</td>
<td>ml/min</td>
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<td>ml/min</td>
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<td>g/lh</td>
</tr>
<tr>
<td>$S$</td>
<td>g$_x$/l</td>
</tr>
<tr>
<td>$t$</td>
<td>min, h</td>
</tr>
<tr>
<td>$t_f$</td>
<td>min</td>
</tr>
<tr>
<td>$T$</td>
<td>$^\circ$C</td>
</tr>
<tr>
<td>vvm</td>
<td>1/lh</td>
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Process Development for Industrial Scale BG Production

\[ \frac{v}{v} \quad - \quad \text{concentration: 'volume per volume'} \]

\[ \frac{v}{v_f} \quad - \quad \text{concentration: 'volume per fermentation volume'} \]

\[ V \quad \text{ml, l} \quad \text{volume} \]

\[ X \quad g_x/l \quad \text{biomass concentration} \]

\[ Y_{x/s} \quad g_x/g_s \quad \text{yield of biomass form substrate} \]

**Greek symbols**

\( \delta \quad \text{min}^{-1} \quad \text{exponential coefficient, } \text{dO}_2 \text{ recovery rate} \)

\( \Delta \varepsilon \quad - \quad \text{dielectric increment} \)

\( \Delta p \quad \text{mbar} \quad \text{pressure loss} \)

\( \varepsilon_0 \quad \text{pF/cm} \quad \text{permittivity of free space} \)

\( \lambda \quad \text{nm} \quad \text{wavelength of light} \)

\( \mu \quad \text{h}^{-1} \quad \text{specific growth rate} \)

\( \mu_{max} \quad \text{h}^{-1} \quad \text{maximal specific growth rate} \)

\( \mu_f \quad \text{h}^{-1} \quad \text{growth rate during feed phase (fed-batch)} \)

\( \sigma \quad \text{var.} \quad \text{standard deviation} \)

**Abbreviations**

3-HPA 3-hydroxypropionic acid

AB antibody

AF anti-foam

APC antigen-presenting cell

API active pharmaceutical ingredient

B batch (fermentation run)

BG Bacterial Ghost

BHV-1 bovine herpesvirus-1

BPL \( \beta \)-propiolactone

cel cells/ml cell counts

cfu cells/ml colony forming units

CSTR continuous stirred tank reactor

dH\textsubscript{2}O deionized water

dO\textsubscript{2} % dissolved oxygen concentration (wrt air saturation)
Glossary

DBM $g_{x/1}$ dry biomass
DF dia-filtration
DNA deoxyribonucleic acid
DMSO dimethyl sulfoxide
EcN *Escherichia coli* Nissle 1917
EcNΔ *Escherichia coli* Nissle 1917 lacking cryptic plasmids pMUT1/2
EcNM522 *Escherichia coli* NM522
EO medium 'Ed Oaks'
EO$^-$ medium 'Ed Oaks', un-buffered
EO$^+(2x)$ medium 'Ed Oaks', buffered, 2-fold concentrated
FB fed-batch (fermentation run)
FCM flow cytometry
FL fluorescence signal
FSC forward scatter
GFP green-fluorescent protein
GMO genetically modified organism
GMP good manufacturing practice
HD high-density
ID mm inner diameter (tube, port)
IM inner membrane
LB low salt Luria-Bertani medium
LB$^+$ Luria-Bertani medium with 1% NaCl
LB$v$ 'vegetarian' low salt Luria-Bertani medium
LE E-lysis efficiency
LI timepoint of E-lysis induction
LPS lipopolysaccharide
MFC mass flow controller
MOMP major outer membrane protein
NC negative control (fermentation run)
nd not determined
OD optical density
OM outer membrane
PCR polymerase chain reaction
PMT photomultiplier tube
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tr>
<td>PMS</td>
<td>process management system</td>
</tr>
<tr>
<td>P/O</td>
<td>promoter/operator system</td>
</tr>
<tr>
<td>PPG</td>
<td>polypropylene glycol</td>
</tr>
<tr>
<td>RFI</td>
<td>radio-frequency impedance</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>real-time</td>
</tr>
<tr>
<td>Sf2a</td>
<td><em>Shigella flexneri</em> 2a</td>
</tr>
<tr>
<td>SNUC</td>
<td>staphylococcal nuclease A</td>
</tr>
<tr>
<td>SSC</td>
<td>side scatter</td>
</tr>
<tr>
<td>STR</td>
<td>stirred tank reactor</td>
</tr>
<tr>
<td>TA</td>
<td>target antigen</td>
</tr>
<tr>
<td>TE</td>
<td>trace element</td>
</tr>
<tr>
<td>TF</td>
<td>Techfors-S</td>
</tr>
<tr>
<td>TFF</td>
<td>tangential flow filtration</td>
</tr>
<tr>
<td>TMP</td>
<td>trans-membrane pressure</td>
</tr>
</tbody>
</table>
Appendix
A.1 Supplementary data for chapter 1

A.1.1 $\beta$-dispersion of cell suspensions

The $\beta$-dispersion of a cell suspension (e.g. *E. coli*) is shown below. The graph is adapted from Carvell et al. [1] identifying the critical frequency $f_c$ and the dielectric increment $\Delta \varepsilon$.

![Figure A.1: $\beta$-dispersion of a cell suspension, adapted [1]](image-url)
A.2 Supplementary data for chapter 2

A.2.1 Pump feed rates for Flowmaster FMT 300

The pump flow rates for the Flowmaster FMT 300 peristaltic pump were recorded for Ismaprene SC0696 tubing (ID = 15.9 mm) as a function of the rotational speed of the pump head. For any desired flow rate \( x \) the corresponding pump speed \( S_p \) can be calculated from:

\[
S_p \text{ (rpm)} = 24.67 \times x + 24.2
\]

The coefficient of determination is \( R^2 = 0.997 \)

Figure A.2: Correlation of pump speed and pump feed rate for Flowmaster FMT 300
A.2.2 ÄKTAcrossflow™ automated filtration system

The system was provided courtesy of GE Healthcare for preliminary experiments towards implementation of a TFF system.

![ÄKTAcrossflow™ automated filtration system](image)

Figure A.3: ÄKTAcrossflow™ automated filtration system

A.2.3 Dia-filtration during batch fermentation of EcN

The following pictures were taken during batch fermentation of EcN in DeLisa medium. The first photo shows the Techfors-S fermenter and the connected TFF module. The second photo shows washing of the inactivated BG product (DF).
Figure A.4: *DF during E-lysis phase for batch production of EcN BGs in DeLisa medium*

Figure A.5: *DF during product refinement (batch production of EcN BGs in DeLisa medium)*
A.3 Supplementary data for chapter 4

A.3.1 Inactivation of EcN (pGLysivb) BGs produced in medium density batch fermentation

In order to determine the amount of DNA that has been withdrawn from the broth by diafiltration an exponential model was used to estimate how much free DNA would have been in the medium without DF. For the medium density batch process with EcN (pGLysivb) E-lysis proceeded exponentially over the first 60 min of E-lysis phase with a rate of $k_l = 5.6 \times 10^{-1}$ h$^{-1}$ [2]. DF was activated 15 min after LI at the anticipated timepoint of E-lysis onset. A mass balance gave that 30 min after LI the purging effect of DF was approximately 15%. With

![Figure A.6: Viability and free DNA for a batch fermentation with EcN (pGLysivb) BG in DeLisa medium](image)

Viability data as obtained from cfu determinations 120 min before and after LI. Free DNA in the culture broth supernatant was measured photometrically (DNA meas). An exponential model was included to estimate the amount of free DNA without purging by dia-filtration (DNA pred). LI: E-lysis induction by T-shift (35 to 42°C)
this information the amount of expelled DNA was estimated (light blue curve in fig. A.6). The model suggested that without DF at the end of E-lysis phase the DNA concentration would be approximately 1,100 µg/ml while the measured concentration of DNA at 120 min was only 528 µg/ml. This would correspond to a reduction of free DNA by 50% before concentration. As these calculations are based on several unconfirmed assumptions the results were revised down by a safety factor of 0.66. This gave a DNA reduction of 33% and with this value the required amount of BPL was calculated to be 0.50% (v/v) for EcN (pGLysivb) BG products obtained from medium density fermentation in DeLisa medium.
A.4 Supplementary data for chapter 5

A.4.1 Batch fermentation of EcN (pGLysivb) BGs in LBv medium

EcN formed cell clusters during growth phase in batch fermentations with LBv. The phenomenon was limited to fermentation of EcN in complex medium and has never occurred during any fermentations with minimal DeLisa medium. The clustering influenced cell count determination; during the first 60 min after inoculation the OD$_{600}$ kept increasing while the cfu dropped. After discovering cell clusters through light microscopy it was concluded that cfu results are misleading for samples taken in that period. Clustering also compromised results obtained via FCM. The cell clusters mimicked larger particles generating accordingly increased FSC signals and also allowed for enhanced DiBAC$_4$(3) accumulation. 2D dotplots of samples taken 30/60 min after inoculation showed a signature tail at the top right hand side of region R1. As for cfu determinations, cell counting by FCM gave misleading results at the beginning of growth phase.

Figures A.7 (a) and (b) show a microscopic picture of a typical cell cluster and a 2D dotplot with the signature tail to region R1, respectively. Usually the clusters disintegrated over the course of the growth phase. After LI no clusters were ever observed and the tail to R1 was 'empty' (fig. A.7 (c)). From this point the cell count results were sound and the LE could be calculated correctly. FCM data obtained from two different 20.0 l batch fermentations (A, B) of EcN (pGLysivb) BGs in LBv are presented in figures A.7 (d) to (g) showing two samples, each, taken 30 min before and 120 min after LI. Both fermentation runs yielded very good LEs as calculated from the cfu (99.994%, 99.990% for A, B, respectively). The dotplots for samples taken 30 min prior to LI show viable populations of EcN with the typical tail to R1. The dotplot representing the end of E-lysis phase in the first example (fig. A.7 (e)), however, indicates insufficient E-lysis performance. A certainly significant fraction of cells came to rest in region R2. In the second example (fig. A.7 (f)) the effect was not as significant. Calculating the LE from FCM data for both runs gave values of 87.319% and 95.674% for A and B, respectively. The values of LE deviate widely from the results determined by cfu.

These findings underline the value of FCM as an additional tool for evaluation of E-lysis performance. Calculating E-lysis efficiencies from cfu data relies on the assumption that the loss of viability for all cells is solely due to E-lysis. Calculating LE from FCM data, however, only regards the fraction of cells that have actually turned into BGs. Therefore, matching
Figure A.7: FCM data for different batch fermentations of EcN BGs in LBv

20.0 l batch fermentation of EcN (pGLysivb) BGs in LBv. (a): light microscopy - 30 min prior to LI. (b): FCM - 30 min prior to LI. (c) FCM - 30 min after LI.
Batch fermentation run A: (d) FCM 30 min prior to LI, (e) FCM 120 min after LI. Batch fermentation run B: (f) FCM 30 min prior to LI, (g) FCM 120 min after LI. LI: E-lysis induction by T-shift (35 to 42°C).

results for LE obtained from both methods is much stronger evidence for successful E-lysis than cfu data alone.

A.4.2 FCM in downstream processing for EcN (pGLysivb) BG production in DeLisa medium

Downstream processing for EcN (pGLysivb) BG production features product recovery via tangential flow filtration. At the end of E-lysis phase the broth is ten-fold concentrated and prepared for lyophilization. FCM was applied during downstream processing to make statements about the quality of the product. Analyzing the product both quantitatively and qualitatively via FCM after downstream processing provides information about possible losses and also whether the product properties have changed. For the latter appearance of the concentrated product was evaluated in 2D dotplots using the same set of polygonal gates.
as for samples taken at the end of E-lysis phase. Figure A.8 compares a sample taken at the end of E-lysis phase (a) with a sample of the ten-fold concentrated product suspension (b). The appearance of the concentrate in the 2D dotplot (b) was very similar to the sample at the end of E-lysis (a) confirming that the product properties had not changed during product recovery. Considering the concentration factor only 79.8% of the total particles were re-discovered in the concentrate.

For later application of freeze-dried BGs the lyophilisate has to be re-suspended. Re-suspension was done by simply adding water or a suitable buffer solution and shaking up the suspension. This procedure should be sufficient to disintegrate BG agglomerates and leave the vast majority of particles singularized. FCM analysis of the re-suspended product was used in order to determine to what degree the BGs are present as individual particles. 1 ml sample of the concentrate was lyophilized, subsequently re-suspended in 1 ml and the treated as a regular FCM sample. Figure A.8 compares the concentrate before lysophilization (b) with a sample of re-suspended lyophilisate (c). The appearance of the re-suspended sample showed that the vast majority of BG particles is found in region R3. The tail at the top right hand side of region R3 was presumably caused by BG agglomerates that had not disintegrated giving higher signals in both the FSC and FL1 channel. Since there was no measure for judging how many particles cohered in the individual agglomerates only the particle counts in region R3 were compared between the re-suspended sample and the the concentrate. Only 58% of the particles present in the concentrate were singularized and

![Figure A.8: FCM for the evaluation of product quality of EcN BGs](image)

Quality considerations for EcNM522 BGs produced in DeLisa medium (2010-11-24): (a) 120 min after LI (end of E-lysis phase); (b) 10-fold concentrated product suspension; (c) re-suspension of the lyophilized product.
re-discovered in the sample of the lyophilisate.

The losses after product recover (> 20%) and especially after lyophilization/re-suspension (overall > 50%) were unacceptable. As handling of highly concentrated BG samples (both fresh and re-suspended) was difficult (quick sedimentation of particles, difficult handling with pipette) the results as shown above may not be conclusive. No reproducible strategy for FCM analysis of samples taken after downstream processing could yet be established. Improving the handling and measurement procedures it should be possible to establish FCM as a reliable tool for evaluation of BG product quality.
A.5 Supplementary data for chapter 6

A.5.1 Batch fermentation of EcN (pGLysivb) BGs in DeLisa medium

The fermentation runs as shown in table A.1 are numbered consecutively (B#). Initial fermentations B01 and 02 represent runs where E-lysis is induced early with a residual glucose of > 12 g/l and without DF during lysis phase. Table A.1 also provides the major results (yield, LEs at 60/120 min) of all batch processes.

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<thead>
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<th>cfu-yield</th>
<th>LE (60’)</th>
<th>LE (120’)</th>
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<td>B01</td>
<td>2.96 · 10^9</td>
<td>99.824</td>
<td>99.830</td>
</tr>
<tr>
<td>B02</td>
<td>3.90 · 10^9</td>
<td>99.737</td>
<td>99.105</td>
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<tr>
<td>B03</td>
<td>9.90 · 10^9</td>
<td>99.720</td>
<td>99.208</td>
</tr>
<tr>
<td>B04</td>
<td>7.60 · 10^9</td>
<td>99.844</td>
<td>99.695</td>
</tr>
<tr>
<td>B05</td>
<td>6.95 · 10^9</td>
<td>99.881</td>
<td>99.711</td>
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<td>6.95 · 10^9</td>
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<td>8.45 · 10^9</td>
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<tr>
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<td>7.55 · 10^9</td>
<td>99.739</td>
<td>99.462</td>
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</table>

Table A.1: Results for batch fermentations of EcN (pGLysivb) BGs in DeLisa medium

1: no DF during E-lysis phase
Cell yield as determined from the cfu (cells/ml), LE at 60/120 min calculated from the cfu.

A.5.2 Establishment of a fed-batch strategy

E-lysis has been reported to be dependent on the physiological state of the culture and is only functional in exponentially growing cells [3, 4]. Therefore it is anticipated that for a fed-batch approach an exponentially increasing feed profile is mandatory and that the feed stream also has to be maintained somewhat beyond the point of LI. In fed-batch fermentation it is common procedure to implement slow growth rates to avoid overflow metabolism, accumulation of unconsumed substrates and possible limitations in aeration. For EcN in unlimited batch growth the maximal growth rate \( \mu_{\text{max}} \) is approx. 0.75 h\(^{-1}\).

A total of 14 fed-batch fermentations were performed. For the first approach with E-lysis
in a fed-batch process (FB01) a growth rate $\mu_f$ of 0.4 h$^{-1}$ was implemented. The glucose concentration in the feed was 250 (g/l), the exponential increasing feed rate was to be maintained during E-lysis phase. However, due to the rapid expulsion of the cytoplasmic contents during E-lysis the viscosity of the culture broth increased abruptly which caused massive foaming. This effect was so drastic that the experiment had to be aborted about 70 min after LI (no results). The following fed-batch runs were setup more cautiously to avoid uncontrollable foaming. Table A.2 shows the consecutive runs (FB#), table A.3 summarizes the major results (cfu, LE) for runs FB02 to 14. Runs FB02 and 03 were performed with a glucose concentration of 100 (g/l) in the feed. Also, the implemented growth rate $\mu_f$ was reduced to 0.25 and 0.35, respectively, while the exponential feed was only to be maintained 30 min into the E-lysis phase. Using 3.0 g/l poly-propylene glycol (PPG) as anti-foam agent in the feed reduced foaming to an acceptable extent. Both FB 02

<table>
<thead>
<tr>
<th>FB#</th>
<th>$S_m$ (g/l)</th>
<th>$\mu_f$ (h$^{-1}$)</th>
<th>$t_f$ (min)</th>
<th>feed profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB01</td>
<td>250</td>
<td>0.4</td>
<td>0'-70'</td>
<td>exp. 1.</td>
</tr>
<tr>
<td>FB02</td>
<td>100</td>
<td>0.25</td>
<td>0'-30'</td>
<td>exp.</td>
</tr>
<tr>
<td>FB03</td>
<td>100</td>
<td>0.35</td>
<td>0'-30'</td>
<td>exp. 2.</td>
</tr>
<tr>
<td>FB04</td>
<td>100</td>
<td>0.35</td>
<td>0'-120'</td>
<td>const.</td>
</tr>
<tr>
<td>FB05</td>
<td>100</td>
<td>0.35</td>
<td>0'-120'</td>
<td>const.</td>
</tr>
<tr>
<td>FB06</td>
<td>100</td>
<td>0.4</td>
<td>0'-120'</td>
<td>const.</td>
</tr>
<tr>
<td>FB07</td>
<td>150</td>
<td>0.4</td>
<td>0'-60'</td>
<td>profile 1</td>
</tr>
<tr>
<td>FB08</td>
<td>150</td>
<td>0.4</td>
<td>0'-60'</td>
<td>const.</td>
</tr>
<tr>
<td>FB09</td>
<td>150</td>
<td>0.4</td>
<td>0'-120'</td>
<td>const.</td>
</tr>
<tr>
<td>FB10</td>
<td>150</td>
<td>0.4</td>
<td>0'-180'</td>
<td>profile 2</td>
</tr>
<tr>
<td>FB11</td>
<td>150</td>
<td>0.4</td>
<td>0'-180'</td>
<td>profile 2</td>
</tr>
<tr>
<td>FB12</td>
<td>150</td>
<td>0.7</td>
<td>0'-120'</td>
<td>profile 3</td>
</tr>
<tr>
<td>FB13</td>
<td>150</td>
<td>0.7</td>
<td>0'-120'</td>
<td>profile 3</td>
</tr>
<tr>
<td>FB14</td>
<td>150</td>
<td>0.7</td>
<td>0'-120'</td>
<td>profile 4</td>
</tr>
</tbody>
</table>

Table A.2: Setup of the fed-batch fermentations for EcN (pGLysivb) BG production

1. heavy foaming - abortion
2. 2nd feed stream (con.) activated after 120'
profile 1: decelerated - 30' con., 30'-60' at 10% con.
profile 2: decelerated - 5' exp., 5'-35' con., 35'-60' at 50% con., 60'-180' at 10% con.
profile 3: decelerated - 5' exp., 5'-35' con., 35'-60' at 50% con., 60'-120' at 10% con.
profile 4: decelerated - 5' exp., 5'-20' con., 20'-40' at 50% con., 40'-60' at 25% con., 60'-90' at 10% con., 90'-120' at 5% con.

exp.: exponential, const.: constant, percentage refers to last exponential value (100%)
and 03 reached similar values for dry biomass (DBM: 25.40 and 24.85 g/l) and cell counts (cfu: \(1.17 \cdot 10^{11}\) and \(8.33 \cdot 10^{10}\) cells per ml) independent from the growth rate. However, halting the feed stream only 30 min after LI resulted in fairly poor LE values at 60' and 120', respectively (97.239 and 98.606\% for FB02 vs. 97.671 and 98.331\% for FB03). Resuming the feed for another 60 min at a constant rate (equal to feed rate at LI) after 120' for FB03 helped to push the LE to 99.768\% at 180'. This was interpreted as the need to maintain feeding throughout all of E-lysis phase.

Consequently, runs FB04 to FB06 were performed with a continuous feed stream during E-lysis phase. The feed rate was frozen at the last value of the exponential feed profile at LI giving decent lysis efficiencies of 99.791, 99.470 and 99.836\%, respectively, after 120'. At the same time the maintained feeding resulted in remarkable accumulation of acetate after LI (> 20.0 g/l after 60') and also unconsumed glucose (~ 5.0 g/l after 120'). These effects were clearly unfavorable and were to be minimized in following runs. Given the fact that in a successful E-lysis process more than 99\% of cells die within the first 60 min it was unlikely that the ongoing conversion of glucose into acetate was done by living cells.

<table>
<thead>
<tr>
<th>FB#</th>
<th>cfu-yield 1.</th>
<th>LE (60')</th>
<th>LE (120')</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB02</td>
<td>(1.17 \cdot 10^{11})</td>
<td>97.239</td>
<td>98.606</td>
</tr>
<tr>
<td>FB03</td>
<td>(8.33 \cdot 10^{10})</td>
<td>97.761</td>
<td>98.331</td>
</tr>
<tr>
<td>FB04</td>
<td>(8.47 \cdot 10^{10})</td>
<td>nd 2.</td>
<td>99.791</td>
</tr>
<tr>
<td>FB05</td>
<td>(8.87 \cdot 10^{10})</td>
<td>99.556</td>
<td>99.470</td>
</tr>
<tr>
<td>FB06</td>
<td>(6.00 \cdot 10^{10})</td>
<td>99.317</td>
<td>99.836</td>
</tr>
<tr>
<td>FB07</td>
<td>(9.73 \cdot 10^{10})</td>
<td>87.507</td>
<td>nd 2.</td>
</tr>
<tr>
<td>FB08</td>
<td>(1.17 \cdot 10^{11})</td>
<td>nd 2.</td>
<td>nd 2.</td>
</tr>
<tr>
<td>FB09</td>
<td>(1.14 \cdot 10^{11})</td>
<td>nd 2.</td>
<td>99.673</td>
</tr>
<tr>
<td>FB10</td>
<td>(6.67 \cdot 10^{10})</td>
<td>94.240</td>
<td>98.150</td>
</tr>
<tr>
<td>FB11</td>
<td>(8.60 \cdot 10^{10})</td>
<td>95.736</td>
<td>98.597</td>
</tr>
<tr>
<td>FB12</td>
<td>(3.67 \cdot 10^{10})</td>
<td>97.038</td>
<td>99.621</td>
</tr>
<tr>
<td>FB13</td>
<td>(1.80 \cdot 10^{10})</td>
<td>97.548</td>
<td>99.863</td>
</tr>
<tr>
<td>FB14</td>
<td>(1.20 \cdot 10^{10})</td>
<td>98.332</td>
<td>99.843</td>
</tr>
</tbody>
</table>

Table A.3: Overview on the results if fed-batch fermentations for EcN (pGLysivb) BG production

1. determined manually, without spiral plater and colony counter
2. \textit{nd}: not determined, e.g. due to sampling issues, overgrown plates

XIV
Moreover, it was suggested that maybe unspecific reactions of cytoplasmic enzymes were responsible (Langemann, Herwig - personal communication). Decelerating the feed profile or curtailing E-lysis phase to 60 min may solve this issue and at the same time save feed volume. The latter was tried in runs FB07 and 08 with 150 g/l glucose in the feed but did not give satisfying results. Therefore, the setup with a constant feed for 120 min was repeated (FB09) with the increased glucose concentration and a growth rate of 0.4 h\(^{-1}\). The cfu-yield for this run was \(1.14 \cdot 10^{11}\) cells per ml (DBM: 29.06 g/l) with a LE of 99.673\% at 120'.

Starting with run FB10 implementing a decelerated feed profile during E-lysis was tested as given in table A.2. Runs FB10 and 11 achieved a reduction of accumulated acetate by approximately 50\% (\(\sim 12.5\) g/l at 60') while LE was not sufficient (98.150 and 98.597\% at 120', respectively). Recalling that E-lysis is best in dividing cells it was suggested that the implemented growth rate \(\mu_f\) should converge to \(\mu_{\text{max}}\) (Langemann, Lubitz - personal communication). Thus, runs FB12 to 14 were performed with a \(\mu_f\) of 0.7 h\(^{-1}\). Runs FB12 and 13 achieved sufficient LEs at 120' of 99.621 and 99.863\%, respectively. Still the results of experiment FB12 showed that the issue of acetate accumulation after LI remained unsolved. For FB13 and 14 no acetate measurements were performed due to a lack of assay.

The gradually decelerated feed profile as described for run FB14 represents the latest stage of development for fed-batch production of EcN BGs. It holds the feed rate constant at the last value at LI for 20 min and then implements a quasi-exponential decline of the provided feed. This approach seizes the findings that the onset of E-lysis usually happens about 20 min after LI and that the progress of E-lysis happens somewhat exponentially.

A.5.3 Dry biomass determination for *E. coli* Nissle 1917 in the fed-batch process

During fed-batch fermentation (runs FB05 to 09) samples were taken to correlate the dry biomass (DBM) with the optical density. The results are plotted in figure A.9. For any measured OD\(_{600}\) x the DBM can be calculated from:

\[
\text{DBM (g/l)} = 0.306 \times - 1.671
\]

The coefficient of determination is \(R^2 = 0.980\)
A.5.4 Correlation of biomass and RFI data for *E. coli Nissle 1917*

During fed-batch fermentation (runs FB03, FB07 to 10) it was possible to correlate RFI data with biomass data obtained from both DBM determination and also cell count data (cfu). The corresponding plots are provided below. Comparing DBM and RFI data (fig. A.10 (a)) the correlation is decent, for any capacity value $x$ the DBM can be calculated from:

$$\text{DBM} (\text{g/l}) = 2.258 \times + 2.837$$

The coefficient of determination in this case is $R^2 = 0.886$. Comparing cfu and RFI data the correlation is not satisfying. Linear regression gives for any capacity value $x$:

$$\text{cfu} (1/\text{ml}) = 7.65E+09 \times - 3.92E+09$$

The coefficient of determination in this case is only $R^2 = 0.749$. 
Figure A.10: **Correlation for RFI data with dry biomass and cfu data for EcN**

(a) Correlation of RFI data with results for DBM (b) Correlation of RFI data with results for cfu.
A.6 Digital data

The attached DVD provides all recorded data; both raw and processed; that is related to the experiments performed during the making of this PhD thesis. All further manual notes are provided in the lab-journals.

A.7 References


Curriculum vitae

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Publications