Doctoral Thesis

In situ product removal as an approach to improve the fermentative production of clavulanic acid

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IN SITU PRODUCT REMOVAL AS AN APPROACH TO IMPROVE THE FERMENTATIVE PRODUCTION OF CLAVULANIC ACID

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Abstract

Clavulanic acid (CA) is a potent β-lactamase inhibitor, which is used clinically in combination with conventional β-lactam antibiotics to treat infections caused by bacteria that would otherwise be resistant to these antibiotics. It is produced on large scale by fermentation of *S. clavuligerus*, however yields are hampered by the simultaneous decomposition of the product. One generic method to prevent the product loss during fermentation is to apply an *in situ* product removal (ISPR) strategy, thereby removing the compound of interest from the vicinity of the cells into a stabilizing environment. In this thesis the potential benefits of ISPR are analyzed with respect to the prevention of product decomposition, but also with respect to inhibitory effects caused by the product or its decomposition products.

To enable the investigation of product inhibitory effects, first a 12-fold parallelized cultivation device was implemented, which allows the continuous measurement of the oxygen transfer rate (OTR), a key parameter for every aerobic culture, based on a respirometric principle. After proving the validity of the applied principle employing *E. coli* cultures growing on defined medium, further optimization steps finally allowed to culture *S. clavuligerus* on a 50mL scale under conditions and with yields comparable to those of an aerated stirred tank reactor. Employing this device, it was shown, that increased levels of CA severely interfered with the physiology of the producing strain at least at and above 1.5 g/L CA, as indicated by a dose-dependent decrease in maximum OTR. However, inhibition of CA synthesis at the level of the current maximum wild-type titer of 0.4 g/L could not be observed,
as CA still continued to accumulate in the medium after addition of CA in this concentration range. Further investigations elucidated that CA itself and not its decomposition products are responsible for the observed drastic effects as the decay products exert a much less severe effect on growing *S. clavuligerus* cultures.

Finally, ion exchange adsorption was examined as a possible method for ISPR. CA could be immobilized effectively on a strongly basic ion exchange resin, but contact times had to be kept short because CA has only limited stability on the resin as well. Thus an ISPR protocol was developed in which CA was removed batchwise from the fermentation broth by treating it twice a day for a limited time with the adsorbent. By this, a total CA concentration of 0.77 g/l could be reached compared to 0.35 g/l in the control culture without ISPR. To compensate for nutrient removal, a soybean extract was intermittently fed and allowed the net formation of CA to further increase to 1.67 g/l, which was 3.2 times higher than in the corresponding control culture representing a clear motivation to pursue ISPR as a technology for yield increase in the CA production process.
Zusammenfassung


Um die produktinhibierenden Effekte untersuchen zu können, wurde zunächst ein 12-fach parallelisiertes Kultivierungssystem in Betrieb genommen, welches die kontinuierliche Messung der Sauerstofftransferrate (OTR) erlaubte. Der OTR ist ein Schlüsselparameter für alle aeroben Kulturen und wurde basierend auf einem respirometrischen Prinzip gemessen. Nach der Validierung des verwendeten Messprinzips mit Hilfe von auf definiertem Medium wachsenden E. coli Kulturen, erlaubten schliesslich zusätzliche Optimierungsschritte die Kultivierung von S. clavuligerus im 50mL Massstab unter vergleichbaren Bedingungen und mit ähnlichen Ausbeuten zu jenen im belüfteten und
gerührtem Reaktor. Mit Hilfe des beschriebenen Messsystems konnte gezeigt werden, dass CA-Konzentrationen von 1.5 s/l und höher die Physiologie des produzierenden Stamms stark beeinträchtigen, was durch die konzentrationsabhängige Abnahme des maximalen OTR's angezeigt wurde. Eine Beeinträchtigung der CA-Synthese im Bereich der maximalen wild-type-Titer von 0.4 s/l konnte jedoch nicht festgestellt werden, da CA im Medium auch dann noch akkumulierte, wenn CA in diesem Konzentrationsbereich zugegeben wurde. Zusätzliche Untersuchungen ergaben, dass CA selber und nicht die Zerfallsprodukte verantwortlich sind für den gemessenen drastischen Effekt, da die Zerfallsprodukte einen viel kleineren Einfluss auf wachsende S. clavuligerus Kulturen haben.

Abschliessend wurde die Ionenaustausch-Adsorption als mögliche ISPR-Methode untersucht. CA konnte effektiv auf einem stark basischen Ionenaustausch-Adsorbens immobilisiert werden, aber die Kontaktzeiten mussten kurz gehalten werden, weil die Stabilität von CA auf dem Austauscher ebenfalls gering war. Deshalb wurde ein ISPR-Protokoll entwickelt, in welchem CA absatzweise aus der Fermentationsbrühe entfernt wurde, in dem diese zweimal täglich mit dem Ionenaustauscher behandelt wurde. Dadurch konnte die Gesamtkonzentration von CA auf 0.77 s/l gesteigert werden, verglichen mit einer Konzentration von 0.35 s/l in einer Kultur ohne ISPR. Um die gleichzeitige Entfernung von Nährstoffen zu kompensieren, wurde periodisch ein Sojabohnen-Extrakt gefüttert, was zu einer weiteren Erhöhung der Nettoausbeute auf 1.67 s/l CA führte. Dies entsprach einer 3.2-fachen Steigerung im Vergleich zu der entsprechenden Kontollkultur und stellt eine deutliche Motivation dar, um zukünftig ISPR-Technologien zur Steigerung der Ausbeute im CA-Produktionsprozess weiter zu entwickeln und einzusetzen.
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Chapter 1

Introduction

The thesis "In situ product removal as an approach to improve the fermentative production of clavulanic acid" deals with the detailed examination of the clavulanic acid (CA) production process by fermentation with special attention to the potential benefits of in situ product removal (ISPR). In the introductory sections, the basic concepts of fermentation and ISPR are summarized, followed by a description of the chosen model compound CA and the outline of the thesis.

1.1 Fermentation processes in biotechnology

Biotechnological processes, employing microbial cells and the enzymes therein, have been used for millenia in the production of food products (Schoemaker et al., 2003). Nowadays, biocatalysis has emerged as an important tool in the industrial synthesis of bulk chemicals, pharmaceutical and agrochemical intermediates and active pharmaceuticals (Panke and Wubbolts, 2005; Schmid et al., 2001). Such processes can be subdivided in two major groups: biotransformation and fermentation. In
biotransformations, a defined starting material is converted with the help of either whole cells or isolated enzymes into the desired product, which typically has a similar chemical structure as the starting material. In fermentations, whole cells are building complex molecules "from scratch" based on simple, cheap, and usually biogenic substrates such as glucose, oils, molasses, or corn steep liquor. A wide range of products, including bulk products such as citric acid, amino acids or ethanol, but also numerous other, rather complex structures such as antibiotics, vitamins, enzymes and pharmaceutical proteins, are obtained by fermentations. If a certain level of productivity is reached, then the benefits of a fermentative process are frequently large (Petersen, 1999) and it is beneficial both from the economic and environmental point of view (OECD, 2001). However, the successful implementation of fermentations is often challenged by the following problems: (i) Certain important production organisms, such as fungi or bacteria of the order actinomycetales, display complex regulation and polymorph growth. Their capacity to produce the compound of interest, typically secondary metabolites, is limited and subject to poorly understood genetic regulation. Typically, complex media are used to reach a certain level of the interesting compound, and the poor chemical definition of the media makes process development and improvements laborious and time consuming. (ii) Furthermore, the product might interfere with its own production, thus limiting final concentrations to low values or (iii) the product might be labile in the fermentation broth and in the presence of cells. The first two issues are currently intensively and successfully addressed by developing superior producing strains employing the ever growing toolbox of recombinant DNA technology, metabolic engineering and synthetic biology (Alper et al., 2006; Ro et al., 2006; Wilkinson and Bachmann, 2006). Alongside to this, also process engineering based methods are rising which foster especially the speed of the bioprocess development phase, which is a key issue in the pharmaceutical industry (Pollard and Woodley, 2007). Laborious medium optimization procedures (see (i)) can now benefit from the growing availability of small scale highly parallel fermentation devices equipped with online data acquisition technology, partly even mimicking production scale conditions (Anderlei et al., 2004; Brethauer et al., 2007; Weuster-Botz, 2005). Finally, the product toxicity and instability issues might be overcome by employing in situ
product removal (ISPR) techniques.

1.2 ISPR as a tool for bioprocessing

ISPR refers to methods for selective removal of products from the vicinity of the biocatalyst as soon as they are formed. This enables (i) to circumvent biocatalyst deactivation by toxic products, (ii) to shift unfavorable reaction equilibria, (iii) to minimize product decay and formation of side products, and (iv) to reduce downstream-processing and polishing steps (Freeman et al., 1993; Lye and Woodley, 1999). General methods employed for ISPR are extraction into a second liquid phase, adsorption on solid sorbents, selective permeation through membranes, crystallization, or evaporation (Freeman et al., 1993). According to the comprehensive review of Stark and von Stockar (2003), more than 50% of the reported ISPR cases made use of extraction methods, whereas adsorption and evaporation covered each almost 20% (Stark and von Stockar, 2003). Analyzing the ISPR cases with respect to the separated product class reveals that organic solvents and biofuels were the target in 37% of ISPR cases. Organic acids constituted the second important product class with a share of 22%, followed by aroma compounds and fine chemicals with each 12% (Takors, 2004). Nowadays, activities in the field of in situ solvent removal are decreasing, whereas aroma compounds and organic acids gaining increasing attention (Stark and von Stockar, 2003).

Although many ISPR processes have proven to be beneficial compared to the standard processes (Lye and Woodley, 1999) and although economic efficiency calculation taking into account the increased complexity of the production facility showed lower overall costs (Daugulis et al., 1991; Takors, 2004), only a very limited number of ISPR processes have been implemented on a commercial scale. Successful in this respect was e.g. the BIOSTIL process, which delivers up to 180,000 L ethanol per day employing in situ evaporation (Takors, 2004). Further examples are the citric and lactic acid production with coprecipitation of their calcium salts and an extractive lactic acid fermentation, commercialized by Cargill Dow Polymers (Stark and von Stockar, 2003). Two examples
in the fine chemistry area, carried out on a preparative scale, involved resin adsorption in a whole cell catalyzed ketone reduction (Vicenzi et al., 1997) or a Baeyer-Villiger oxidation (Alphand et al., 2003; Hilker et al., 2004, 2005). Both cases concern biotransformations, where the adsorbent not only served as a product sink but also as a reservoir for the toxic substrate.

A number of recent studies suggests that ever more products in the pharmaceutical, fine chemical, and bulk chemical area will be produced by biocatalytic processes (CEFIC, 2004; Dechema, 2004; OECD, 2001). Many of those will encounter the above mentioned issues of biocatalyst inhibition, product instability or unfavorable reaction equilibrium (Takors, 2004) and the full potential of ISPR in these cases is not yet explored. The continuing development of more robust, scalable and flexible ISPR processes and their proof of success in pilot plants will subsequently lead to more industrial ISPR processes, which will gain momentum after the "first of its art"-hurdle in the respective companies is overcome - as it was also the case for the implementation of biotransformations in the chemical industry (OECD, 2001).

1.3 The $\beta$-lactamase inhibitor clavulanic acid

The discovery of $\beta$-lactam antibiotics such as penicillins and cephalosporins represents one of the most important medical breakthroughs of the last century and enabled the development of effective drugs for treating various bacterial infections. This type of antibiotics inhibits transpeptidase, an enzyme involved in forming the bacterial cell wall, and thereby initiates cell death. However, certain bacterial strains have evolved effective defense mechanism against the antibiotics by expressing $\beta$-lactamase. The clinically most important $\beta$-lactamases belong to the class of serine acylases and are able to catalyze the degradation of the $\beta$-lactam antibiotic (Fig. 1.1 a) (Lin et al., 2002).

However, this step can be effectively suppressed. In a screening program for $\beta$-lactamase inhibitors, initiated by the Beecham Laboratories (Great
Figure 1.1: a) Schematic diagram showing the inhibition of penicillin-G by β-lactamase (E'). b) Schematic diagram showing the inhibition of β-lactamase by CA.
Britain), clavulanic acid (CA) was discovered in the supernatant of a \textit{Streptomyces clavuligerus} cultivation. It is a bicyclic $\beta$-lactam with 2R, 5R stereochemistry. The nucleus resembles that of penicillins, but the five-membered ring contains oxygen instead of sulfur and lacks a C6 side chain (Fig. 1.2) (Brown et al., 1976).

This molecule is able to inhibit $\beta$-lactamases very effectively in an irreversible manner (Fig. 1.1 b). CA itself possesses only weak, though broad-spectrum, antibiotic activity, but the co-formulations with other $\beta$-lactam antibiotics such as amoxicillin are very effective against numerous infections caused by $\beta$-lactamase producing pathogens and are on the market since 1981. These combination drugs are prescribed in more than 150 countries and have attained sales in excess of 2 billion dollars yearly (Li and Townsend, 2006). Interestingly, resistance development to these combinations drugs is minimal (Demain and Elander, 1999).

The producing strain \textit{S. clavuligerus}, an isolate from a South American soil sample, produces not only CA as secondary metabolite, but also, amongst others, cephamycin C, isopenicillin N, deacetylcephalosporin C and several clavam compounds. The clavam compounds differ from clavulanic acid in lacking the C2-carboxyl group and in having 5-S stereochemistry (Liras and Rodriguez-Garcia, 2000).

The genes for the biosynthesis of these compounds are arranged in a super-cluster on the genome of \textit{S. clavuligerus} (MacKenzie et al., 2007). Approximately 18 genes (orf2 - 19) are assumed to encode proteins involved in the biosynthesis, transport and regulation of CA.

![Figure 1.2: The chemical structure of CA.](image)
The biosynthesis is initiated by the condensation of L-arginine and D-glyceraldehyde-3-phosphate, followed by further steps to clavaminic acid. Clavaminic acid serves as a branch point between the biosynthesis of CA and other clavams that have the 5-S stereochemistry. In the final steps of CA biosynthesis, clavaminic acid undergoes a double epimerization and oxidative deamination to yield clavulanate-9-aldehyde, which is then converted in an NADPH-dependent reaction to CA (Fig. 1.3) (MacKenzie et al., 2007). On industrial scale, CA is produced in 100 h fermentations of *S. clavuligerus* on a complex medium with soybean protein as a key component and glycerol, soluble starch or lipids as carbon source at 27°C and pH 6.5 - 7 (Elander, 2003). The producing strain shows a filamentous growth with a morphology ranging from pellets to free mycelia. Recent industrial production data are scarce and final CA titers are not disclosed, with the exception of one patent, claiming a yield of 3.8 g/L (Cardoso, 2002).

During the fermentation, the product decays concomitantly with its production. The decomposition is accelerated during fermentation by the bacteria itself or compounds produced by the cells (Roubos et al., 2001). The generally poor chemical stability also makes the downstream processing of this compound troublesome, which usually involves various extractions with butanol or ethylacetate under acidic conditions and ion exchange chromatography as part of a multi-step protocol at reduced temperature, resulting in rather poor yields of typically around 40% (Butterworth, 1984).
Figure 1.3: Biosynthesis of CA (adapted from (MacKenzie et al., 2007)). Abbreviations used are CeaS: N2-(2-carboxyethyl)arginine synthase; BLS: \(\beta\)-lactam synthetase; PAH: proclavaminate amidinohydrolase and CAS: clavaminic acid synthase.
1.4 Scope of the thesis

The β-lactamase inhibitor CA is produced on large scale by fermentation of *S. clavuligerus*. Yields are hampered by the simultaneous decomposition of the product. This suggests that ISPR should have a strong impact on product yields, if a method is found that can stabilize the product. Thus, this thesis examines the potential of ISPR to improve the process, not only with respect to product decomposition, but also to product and decomposition product inhibitory effects on the cells. Chapter 2 describes the investigation of the CA degradation kinetic, which revealed a so far unnoticed self-catalytic effect of CA on its own decomposition. Chapter 3 describes the basic work necessary for the product inhibition examinations by modifying, characterizing and validating a 12-fold parallel, respirometry-based oxygen transfer rate (OTR) measuring device, the Sapromat E, with the aid of *E. coli* cultivations. In chapter 4 the effect of CA on its producing strain is examined, after further optimizing the Sapromat E for cultivations of *S. clavuligerus* in rich production medium, followed by the investigation of the influence of CA decomposition products on *S. clavuligerus*, as described in chapter 5. Finally, in chapter 6, the application of ISPR by ion exchange adsorption in *S. clavuligerus* fed batch fermentations is described. Chapter 7 summarizes the results and presents an outlook.
Chapter 2

The hydrolysis of clavulanic acid is catalyzed by the compound itself and by its decomposition products

Abstract

In the presented work, the decomposition kinetic of the β-lactamase inhibitor clavulanic acid (CA) was observed for CA concentrations between 2.5 and 20 s/L, which is assumed to represent a characteristic range for an industrial CA production process. For each initial concentration, first order kinetic plots could be obtained, however the kinetic constant
increased from 0.0038 to 0.0086 1/h with increasing initial CA concentration. We concluded that CA accelerates its own decomposition by general acid-base catalysis. As the kinetic constant remained unaltered during the reaction, it was reasoned that also the decomposition products of CA have to show similar catalytic activity, which was confirmed experimentally by showing that CA decomposition rates increased when CA degradation products were added to the reaction. A kinetic model was proposed, which was able to reliably predict the observed pseudo first order rate constants. Furthermore we could show that the composition of the decay products mixture was altered with varying CA concentrations. The presented results reinforce the requirement to pay special attention with respect to further conditions such as pH and temperature if high CA concentrations are employed to avoid the excessive decomposition of the compound.

2.1 Introduction

Clavulanic acid (CA) (1) ((2R,3Z,5R)-3-(2-hydroxyethyliden)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptan-2-carboxylic acid) is a fused bicyclic \(\beta\)-lactam molecule produced by *Streptomyces clavuligerus*, a microbial isolate from a South American soil sample. CA is a potent inhibitor of \(\beta\)-lactamases produced by some strains of pathogenic microorganism resistant to \(\beta\)-lactam antibiotics thereby enabling efficient treatment of infectious diseases (Reading and Cole, 1977) and is one of the active ingredients in Augmentin — a commercially highly successful antibiotic medicine. The production as well as the downstream processing is accompanied by the compound’s degradation by hydrolysis, which is around 10 times faster than the one of penicillin G if the lowest rates at the optimal pH value are compared (Haginaka et al., 1981). Therefore a considerable amount of the substance gets lost during the production process.

Several studies examined various aspects of the decomposition of CA: the mechanism, final degradation products (Finn et al., 1984; Haginaka et al., 1985), and the decomposition kinetics in different buffered solutions or fermentation broth (Haginaka et al., 1981; Ishida et al.,
Figure 2.1: Clavulanic acid (1) and its major decomposition products: 1-amino-2-oxo-butan-4-ol (2), 2,5-bis(2-hydroxyethyl)pyrazine (3), 3-carboxyethyl-2,5-bis(2-hydroxyethyl)pyrazine (4) and 3-ethyl-2,5-bis(2-hydroxyethyl)pyrazine (5).

2006; Mayer and Deckwer, 1996; Roubos et al., 2002). The results included that the hydrolysis of CA under neutral or basic conditions ultimately leads via the reactive amino ketone (2) to the pyrazines (3 - 5) (Fig. 2.1) (Finn et al., 1984; Haginaka et al., 1985). However, the pyrazines (3 - 5) are not the only decomposition products, Finn et al. (1984) stated "complex product mixtures" and very low total pyrazine yields of only 20% (Finn et al., 1984). Haginaka et al. (1985) studied the stability of CA in different buffers over a pH range of 3 - 10 at 35°C. They found that the degradation followed pseudo first-order kinetics and was catalyzed by various buffer salts. The rate constants in the alkaline regions were about ten times larger than in acidic solutions (Haginaka et al., 1981). Not only buffer salts, but also production medium ingredients, metabolites of *S. clavuligerus* cells, amino acids and different metal chlorides were found to influence the observed
decomposition rates (Bersanetti et al., 2005; Ishida et al., 2006; Martin et al., 1989; Mayer and Deckwer, 1996; Roubos et al., 2002).

While investigating the effect of CA on the producing strain, we found indications that the decomposition kinetic might also be dependent on the CA concentration. This is of importance, as the concentration of CA in solution during its life span from fermentative production over the downstream processing to its final application as a drug ingredient covers a wide range. In fermentations of the wild-type strain, final titers of 0.5 g/L are reached (Gouveia et al., 2001; Mayer and Deckwer, 1996; Roubos et al., 2002), however in industrial fermentations employing overproducing mutants much higher concentrations of up to 10 g/L are expected. Such data are usually proprietary, but e.g. a final titer of 3.8 g/L was disclosed in a patent, supporting the statement above as well as concentrations of 10 - 30 g/L during downstream processing (Cardoso, 2002). Furthermore, Augmentin is partly marketed as a suspension - e.g. Augmentin ES-600 contains after reconstitution 8.6 g/L CA. However, an analysis of the available literature showed, that the presented kinetic investigations regarded only a concentration range of 0.04 - 2.5 g/L. In this work we investigated the decomposition kinetics for CA concentrations of up to 20 g/L, covering thereby a concentration range of practical importance.
2.2. MATERIALS AND METHODS

2.2. Materials and methods

2.2.1 Cultivation of *S. clavuligerus*

First precultures of *S. clavuligerus* (DSM 738, DSMZ, Braunschweig, Germany) were inoculated with 100 µL spore suspension from a deep frozen stock (-20°C) and grown on 20 mL ISP1 medium (tryptone 5 g/L, yeast extract 3 g/L, adjusted to pH 7 with NaOH) at 30°C on an orbital shaker at 250 rpm for 24 h. An aliquot of 1.85 mL of this culture was added to 35 mL of production medium (glycerol 20 g/L, soybean extract 200 mL/L, peptone 5 g/L) in a 500 mL Erlenmeyer flask stirred with 280 rpm with a 50 mm stirrer bar (Komet 50, H+P Labortechnik, Munich, Germany). Soybean extract is the supernatant of an autoclaved 60 g/L soybean-flour suspension.

2.2.2 Decomposition of CA in *S. clavuligerus* fermentations

Cultures of *S. clavuligerus* in production medium were supplemented at inoculation (t = 0 h) with different volumes of a sterile filtered stock solution containing 60 g/L potassium clavulanate (International Laboratory, San Bruno, USA) in production medium and incubated as described above. At regular intervals, samples were withdrawn from the culture and the supernatant was either directly used for analysis or stored at -20°C until analysis.

2.2.3 Influence of [CA]₀ on CA decomposition kinetic

10 mL of solutions containing different amounts of potassium clavulanate in 0.1 M 3-(N-morpholino)propanesulfonic acid (MOPS) buffer pH 7 were shaken at 28°C in 15 mL closed falcon tubes. Aliquots of 0.5 mL were removed and the remaining CA concentrations were measured immediately. All experiments were performed in triplicates. The average
values of each three measurements were used to calculate the decomposition constant by a curve fit with the IGOR software (Wavemetrics, Inc., Portland, OR, USA).

2.2.4 Influence of CA decomposition products on CA decomposition kinetic

A solution of potassium clavulanate in 0.1 M MOPS buffer pH 7 containing 20 mg/L CA was incubated at 80°C for 48 hours and was thereby completely degraded. After cooling, this solution was diluted 1:2, 1:4, and 1:20 with 0.1 M MOPS buffer and potassium clavulanate was added to final CA concentrations of 5 mg/L. Decomposition rate constants were obtained as described above.

2.2.5 Influence of EDTA on CA decomposition kinetic

Potassium clavulanate was dissolved to a final CA concentration of 2 mg/L or 17.6 mg/L in 0.1 M MOPS buffer (pH 7), 0.05 equivalents EDTA were added and decomposition rate constants were obtained as described above.

2.2.6 Decomposition product pattern of CA hydrolysis

Solutions of potassium clavulanate in 0.1 M MOPS containing 2.5, 10 or 20 mg/L CA were shaken at 28°C for 21 days. The resulting mixture of decomposition products was analyzed by HPLC (see below).

2.2.7 Analytical methods

Prior to analysis, the supernatant of a sample was mixed with an imidazole reagent (206 mg/L of imidazole in water, adjusted to pH 6.8 with 5 N
HCl) in a volume ratio of 4:1 (Foulstone and Reading, 1982). Then, the CA in the sample was quantified as the imidazole derivative by HPLC on a Merck LaChrom system L-7200 (VWR, Dietlikon, Switzerland) equipped with a diode array detector (Merck LaChrom L-7455) employing a Prontosil Eurobond C18 5.0 µm column (Bischoff Chromatography, Leonberg, Germany) as stationary phase. As mobile phase served a mixture of 30% methanol and 70% 50 mM KH₂PO₄ (pH 4.5). UV-detection at 312 nm was used to record elution volume.

Decomposition products were analyzed with the above mentioned HPLC system employing 0.1% TFA (solvent A) and acetonitrile (solvent B) as eluents. The following pump program, always with linear gradients, was used for separation: 0 - 1 min: 100% A; 1 - 6 min: 100% A to 95% A / 5% B; 6 - 17 min: 95% A / 5% B; 17 - 24 min: 95% A / 5% B to 100% B; 24 - 34 min: 100% B; 34 - 35 min: 100% B to 100% A; 35 - 40 min: 100% A. The detector wavelength was set to 278 nm. After HPLC separation, the eluted material was fractionated and peaks were identified by MS (Finnigan MAT TSQ 7000, Thermo Electron, Waltham, USA) and NMR (Mercury-vx 300, Varian, Palo Alto, USA) analysis. The following known decomposition products of CA were identified: i) 2,5-bis(2-hydroxyethyl)pyrazine (3) (MH⁺ 169; δ (CD₃OD) 2.98 (4H, t, 6.5 Hz), 3.9 (4H, t), 8.45 (2H, s)), ii) 3-carboxyethyl-2,5-bis(2-hydroxyethyl)pyrazine (4) (MH⁺ 241; δ (CD₃OD) 2.81-3.23 (8H, m), 3.9 (4H, m), 8.25 (1H, s)) and iii) 3-ethyl-2,5-bis(2-hydroxyethyl)pyrazine (5) (MH⁺ 197; δ (CD₃OD) 1.28 (3H, t, J 8 Hz), 2.86-3.05 (6H, m), 3.90 (4H, t, J 6.5 Hz), 8.30 (1H, s)).

2.3 Results

In the presented work, we investigate the influence of the CA concentration on its decomposition kinetic for concentrations between 2 and 20 s/l. The origin of this investigation were experiments regarding the influence of CA on the producing strain S. clavuligerus. Different amounts of CA were added to a freshly inoculated culture of S. clavuligerus and the time course of CA concentration was followed (Fig. 2.2). The hydrolysis of
CA is usually described by a first order kinetic of the form

\[
\frac{d[CA]}{dt} = -k_{obs} \cdot [CA] \, .
\] (2.1)

Thus, fitting an exponential curve to the plot \([CA][CA]_0^{-1}\) versus time yields the first order decomposition constant \(k_{obs}\) (Bersanetti et al., 2005; Ishida et al., 2006; Mayer and Deckwer, 1996; Roubos et al., 2002). Surprisingly, the decomposition of CA in the solution containing originally 4 g/L is faster than in the less concentrated one, the decomposition constant determined by the exponential fit is 2.75 times higher than in the 0.5 g/L solution (Tab. 2.1), which is not the expected behavior for a first order reaction. As conditions change constantly over a cultivation due to the consumption of nutrients, the production of biomass, and the excretion of by-products, we reinvestigated the influence of CA on its own decomposition kinetic in defined buffered solutions instead of cultivation medium.

The effect of an acceleration of the decay CA by the compound itself
2.3. RESULTS

Table 2.1: Summary of the observed kinetic constants for the decomposition of CA under different conditions.

<table>
<thead>
<tr>
<th>Decomposition background</th>
<th>[CA]₀ /g/L</th>
<th>kₜ₁₀₀ /⁻¹/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growing <em>S. clavuligerus</em> culture</td>
<td>0.5</td>
<td>0.008 ± 0.0003</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.022 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.0038 ± 0.0001</td>
</tr>
<tr>
<td>0.1 M MOPS buffer, pH 7</td>
<td>10</td>
<td>0.0061 ± 0.0003</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.0086 ± 0.0006</td>
</tr>
<tr>
<td>0.1 M MOPS, 0.05 eq EDTA, pH 7</td>
<td>2</td>
<td>0.004 ± 0.0002</td>
</tr>
<tr>
<td></td>
<td>17.6</td>
<td>0.0076 ± 0.0003</td>
</tr>
<tr>
<td>Decomp. products from 2 g/L CA</td>
<td></td>
<td>0.0044 ± 0.0002</td>
</tr>
<tr>
<td>Decomp. products from 5 g/L CA</td>
<td>5</td>
<td>0.0049 ± 0.0001</td>
</tr>
<tr>
<td>Decomp. products from 10 g/L CA</td>
<td></td>
<td>0.0067 ± 0.0003</td>
</tr>
</tbody>
</table>

could be reproduced in 0.1 M MOPS buffered solution (Fig. 2.3). The decomposition constant increased from 0.0038 ¹/h to 0.0086 ¹/h by applying higher starting concentrations (Tab. 2.1). This implies that there has to be a concentration depended catalytic effect of one compound in the solution leading to an acceleration of the reaction at higher starting concentrations. The experiment also showed that the rate constant remained constant over the investigated period, the lines in the logarithmic plot are even crossing each other (Fig. 2.3 b), showing that the catalytic effect remained constant during the reaction.

Next, we examined the possible reasons for this unexpected kinetic behavior. One possible explanation could be that the reaction is accelerated by metal impurities in the CA salt. Therefore, we repeated the experiments with 0.05 eq EDTA present in the solution, which masks divalent metal ions by complexation (Martin et al., 1989). However, the same effect as in solutions without EDTA could be observed (Tab. 2.1).

Alternatively, CA could itself catalyze its own decomposition. How-
Figure 2.3: Decomposition of CA in MOPS buffer plotted a) as the course of relative CA concentration with time and b) in the logarithmic form.

ever, as the catalyst is then consumed during the reaction, this would be in contrast to the observation that a pseudo-first order degradation constant remains essentially constant over the degradation process (see Fig. 2.3 b). Consequently, this requires the assumption that the CA degradation products have to show a similar catalytic activity to ex-
2.3. RESULTS

Figure 2.4: Influence of different amount of CA decomposition products (DP) added at \( t = 0 \text{ h} \) to the reaction mixtures with \([\text{CA}]_0 = 5 \text{ g/L}\).

Table 2.2: Fractions of pyrazines (3), (4), (5) and the unknown decomposition product (mix) 6 based on HPLC areas in the degradation mixtures of CA as a function of CA starting concentration.

<table>
<thead>
<tr>
<th>([\text{CA}]_0 \text{/g/L})</th>
<th>(3)/%</th>
<th>(4)/%</th>
<th>(5)/%</th>
<th>(6)/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>26</td>
<td>6</td>
<td>45</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>3</td>
<td>24</td>
<td>38</td>
</tr>
<tr>
<td>20</td>
<td>17</td>
<td>3</td>
<td>27</td>
<td>53</td>
</tr>
</tbody>
</table>

plain the observed behavior. In order to test this hypothesis, a stock solution of degraded CA was diluted to different concentrations between
2 and 10 g/L with MOPS buffer and CA was added to a final concentration of 5 g/L. Indeed, we could confirm that increased amounts of degraded CA in the solution led to higher measured decomposition rates (Fig. 2.4, Tab. 2.1).

Finally, we investigated whether the final decomposition product pattern changed with increased reaction velocity. To this end, we analyzed the final decomposition product pattern by means of HPLC. The chromatograms (Fig. 2.5) showed a complex mixture of decomposition products, from which some peaks could be assigned to the known decomposition products (see Materials and Methods). However, the substance or substances eluting after 21 min have not yet been identified. Analyzing the relative composition of the mixture based on the observed areas of the four main peaks indicated for increasing initial CA concentrations the increasing repression of the formation of the pyrazines 3, 4 and 5 in favor of the unknown substance or mixture of substances 6 (Tab. 2.2).

![Chromatogram of a completely degraded CA solution. The peaks 3 - 5 have been identified as pyrazines (3) to (5), but the nature of peak 6 is unknown.](image)
2.4 Discussion

The decomposition of CA can be satisfactorily described by pseudo first order kinetics, as shown by several literature data and also confirmed by our own measurements. However, our data showed that the observed pseudo first order decomposition constant depends on the initial CA concentration, a fact which was not yet accounted for in the available literature. Based on the presented data, we therefore hypothesized that CA and its degradation products catalyze the compound’s hydrolysis. Similar observations were made for other antibiotics, e.g. the hydrolysis of ceftazidime (Fubara and Notari, 1998) is self-accelerated at higher concentration because ceftazidime and its decomposition product act as similarly strong general base catalysts. The same reaction is catalysed by HPO$_4^{2-}$ ions acting also as general base catalyst. The authors thus concluded, that all antibiotics, which are sensitive to general acid-base catalysis and contain functional groups such as amino- or hydroxyl groups which can act as such catalysts, are likely to self catalyze their own decomposition (Fubara and Notari, 1998). Indeed, the isomerisation of ceftibuten is facilitated by itself as a general acid or base catalyst (Hashimoto and Hirano, 1998), so is the hydrolysis of amoxicillin by the catalytic action of the phenolic hydroxyl group (Bundgaard, 1977) and the degradation of imexon (Kuehl et al., 2006). Literature data showed that CA degradation is prone to general acid or base catalysis, as the observed decomposition constants vary with buffer and medium components whereas amino- and ammonium groups have the most pronounced effect (Haginaka et al., 1981; Ishida et al., 2006; Martin et al., 1989; Mayer and Deckwer, 1996; Roubos et al., 2002). Therefore, we reasoned that it is highly likely that CA as well as its decomposition products, both containing hydroxy- and amine groups, act as catalysts for the hydrolysis.

With this information we proposed a kinetic model which expresses $k_{obs}$ as a function of the catalyst concentration, as often applied for general acid-base catalysis:

$$k_{obs} = k_{MOPS} + k_{cat}([CA]_0 + [DP]_0) \quad .$$ (2.2)

The catalytic constant $k_{MOPS}$ denotes the decomposition constant in the
absence of any other catalytic species except MOPS (0.1 M, pH 7) and is the catalytic constant for the sum of CA and decomposition products (DP) at $t = 0$. The decomposition product term is included here to be able to model those experiments for which decomposition products were added at the beginning of the reaction. In order to test this model, we combined equation 1 and 2 and fitted the resulting equation to our complete data set in a global manner to obtain the values for $k_{MOPS} ((3.01 \cdot 10^{-3} \pm 0.23 \cdot 10^{-3}) \text{ h}^{-1})$ and $k_{cat} ((2.58 \cdot 10^{-4} \pm 0.23 \cdot 10^{-4}) \text{ L gh}^{-1})$ and compared the resulting $k_{obs}$ with the measured decomposition constant (Fig. 2.6). The measured values were in agreement with the modeled data. However, the points for experiments without degradation products at the beginning lay in the upper range of the calculated area, whereas the values for the reactions supplemented with degradation product lay in the lower range. This suggests that as degradation progresses the catalytic activity of the resulting compound mixes decreases.

Although the described model is able to predict the kinetic of the re-
action in a rather reliable manner, it appears important to mention the limits of the chosen approach, which assigns only one catalytic constant to CA and its degradation products. Obviously, this approach does for example not appreciate in detail the observation that the decomposition product pattern changes (Tab. 2.2). A more comprehensive model would need to include the kinetic of further reaction steps to the final products including the unknown mixture 6, which are likely to be catalyzed by the various substances as well, as indicated (based on HPLC peak area assignments) by the increasing fraction of the unknown compounds (eluting later than 20 min under the chosen conditions) with increasing initial CA concentrations.

The presented results reinforce the need to work at low concentration or to take special attention to otherwise optimal conditions such as pH and temperature to avoid the decomposition if high CA concentrations are employed.
Chapter 3

Online medium-throughput respirometry based OTR measurements in magnetically stirred cultures

Simone Brethauer, Martin Held, Sven Panke; Biotechnology and Bioengineering (2007), in press
Abstract

Intensified bioprocess development requires parallelized medium- to high-throughput experimentation with high on- and offline data density across all early scales of the development trajectory from microtiter plate via shake flask to lab-scale reactor. We developed a wide-spread measurement principle for intermediate scales, respirometry, into a parallelized oxygen transfer rate measurement device that could accurately record common process development-relevant effects such as acetate formation, diauxic growth, and nutrient limitations. The device was further equipped with dissolved oxygen measurement capability and sampling ports that allowed repetitive monoseptic sample withdrawal without disturbing the cultivation. Optimization of the operating parameters lead to $k_L a$ values of up to $160 \text{ h}^{-1}$ and corresponding oxygen transfer rates of $1 \text{ g/L/h}$ for cultivation volumes of $50 \text{ mL}$.

3.1 Introduction

Modern bioprocess development takes advantage of advances in microreactor and sensor technology in order to provide data for ever more key process development parameters on ever smaller scales. Consequently, high-throughput cultivations including the online measurement of pH, optical density (OD) and dissolved oxygen tension (DO) can be carried out in volumes below 1 mL (Doig et al., 2005a; Kostov et al., 2001; Maharbiz et al., 2004; Szita et al., 2005), reducing considerably the time and resources required for a first screen of possible parameter variations. This tendency to smaller cultivation devices is complemented by the requirement to increase the data density also at intermediate cultivation scales, such as shake flasks (Anderlei and Buchs, 2001; Betts et al., 2006; Frachon et al., 2006; Puskeiler et al., 2005). Data such as cell dry weight concentration (measured as OD), nutrient, substrate or (by-)product concentrations, and aeration status of the culture and the corresponding oxygen consumption rate are highly desirable, but rarely available on- or at-line from one cultivation vessel in a parallelizable format. In particular information on the aeration status of the culture is
helpful in evaluating the physiological state of aerobically growing microbial cultures. Numerous parameters such as metabolic activity of non-growing cultures, growth kinetics, substrate or product inhibitory effects, and substrate limitations can be studied on the basis of DO or oxygen transfer rate (OTR) measurements, which is why online DO measurements and off-gas analysis are key process parameters in any aerobic biotechnological process once it has reached reactor-scale (Anderlei and Buchs, 2001). While at small cultivation scales availability of DO measurements alone might be sufficient to detect promising clones or exclude experiments that are unsuitable for evaluation (for example because the results have been influenced by a temporary oxygen limitation), it is desirable to complement these data with OTR measurements at intermediate cultivation scales, because the OTR allows proper balancing of the cultivation experiments and thus to obtain vital data for further process development. DO-measurements based on oxygen optodes can now easily be implemented down to small vessel volumes (below 1 mL). OTR measurements require at least shake flask size. Available parallelizable technology (Anderlei et al., 2004) relies on monitoring the drop in oxygen concentration in the closed vessel over a measurement phase to calculate the OTR, followed by a purge phase to increase oxygen concentration again. An additional differential pressure sensor allows the calculation of the carbon dioxide transfer rate from the same data. The complete measurement cycle takes around 30 min, although it remains unclear whether it could be reduced. Next to this recent development, devices for parallelized measurements of oxygen demand for environmental samples based on respirometry have been available for quite some time (Ros et al., 1990; Verstrae et al., 1974). These devices are widely used in the scientific community, inexpensive, and robust. The measurement principle is simple: the oxygen partial pressure drops upon oxygen consumption for respiration or growth. This pressure drop can be detected by a differential manometer and compensated by electrochemically produced oxygen, which is the parameter that is actually measured. The carbon dioxide that is produced from respiration and that would interfere with the measurement is captured by an adsorbent in the gas space of the cultivation vessel. In this work, we describe our efforts to implement a respirometry based parallelized cultivation device to support bioprocess development for rapidly growing aerobic cultures. The result-
ing device allowed online measurement of DO, OTR, and sampling for offline measurements of cell dry weight concentration, pH and compound concentrations from the same flask.

3.2 Materials and methods

3.2.1 Sapromat E

We selected the Sapromat E (H+P Labortechnik AG, Oberschleissheim, Germany) for our activities. It consists of 12 magnetically stirred 500 mL flasks which are placed in a closed water bath to ensure a constant temperature. The stirrer speed in the original system is fixed to 280 rpm. Each flask is connected to a carbon dioxide trap, a switch manometer and an electrochemical oxygen production unit thereby forming a closed system (Fig. 3.1). As discussed above, the measured parameter is the electrochemical production of oxygen. In the Sapromat, oxygen production is discretized. One production cycle lasts 36s at a pre-adjusted production rate, resulting in production of between 0.025 and 0.5 mg of oxygen per cycle. Accordingly, the maximum possible oxygen production is $50 \text{ mg/h}$. Data are recorded via a computer software (Sapromat E steering software 6.0.2), which records the cumulated oxygen production and saves it as a function of time with 100 points per hour.

3.2.2 Modifications of the Sapromat E

To enable variable and high stirring speeds between 100 and 2000 rpm, an external electrical control unit (Telemodul 40C, H+P) was implemented. The original flasks were replaced by 500 mL Erlenmeyer flasks (Schott Schweiz AG, St. Gallen, Switzerland) which had a sufficiently flat bottom to sustain increased stirring rates. To aerate the cultures, cylindrical magnetic stirrer bars of 8 mm diameter and 40 mm length with a samarium cobalt core inducing a strong magnetic field (Semadeni, Ostermundingen, Switzerland) were used. Lithium zirkonate (Fluka, Buchs,
Figure 3.1: Schematic drawing of a Sapromat measuring unit with the following parts: 1: magnetic stirrer, 2: culture flask, 3: carbon dioxide trap, 4: oxygen production flask, 5: switch manometer, 6: control unit for 12 measuring devices connected to a PC.

Switzerland) was used as carbon dioxide sorbent unless mentioned otherwise. The entire system was kept at a constant environmental temperature of 37°C unless otherwise mentioned. Due to the stirring, the temperature of the cultivation flask was constantly approximately 1°C higher than the surrounding temperature, as shown by frequent temperature measurements over several experiments. This temperature difference led to a slight overpressure in the reaction flask due to the higher water vapor pressure. This overpressure reproducibly corresponded to three oxygen production cycles, which are missing as a small systematic mistake at the beginning of all measurements.
3.2.3 OTR calculation

The output of the Sapromat software is the cumulated oxygen consumption in the form of a compensating oxygen production rate step function with one measuring point every 36 s. As the oxygen production rate had to be pre-adjusted, only two states are possible - oxygen production or no production. This digital step function was transformed to an analog function by a routine programmed in IGOR (WaveMetrics, Inc., Portland, USA). The point of origin and the vertices of the step function were selected, and new points between these vertices were calculated by linear interpolation, thereby creating a new data file with the same number of measurement points. To smooth out measurement noise, these new values were averaged over a sliding window of a time span of 360 s, and the average value was ascribed to the time in the center of the window. OTRs were calculated as the first derivative of this cumulated oxygen consumption curve. This leads to a systematic underestimation of the oxygen consumption for the first oxygen production cycle. Consequently, we started plotting OTR curves only after the first production cycle.

3.2.4 Measurement of dissolved oxygen

Dissolved oxygen levels were measured with an optical system (Presens GmbH, Regensburg, Germany). A sensor spot containing a fluorescent dye was immobilized on the bottom of the measuring flask and a polymethylmethacrylate optical fiber was placed underneath the flask in the water bath of the Sapromat. The optical fiber was connected to the four channel oxygen meter Oxy4, which was further connected to a PC. The measuring principle is based on the dynamic quenching of luminescence by oxygen molecules (Klimant et al., 1995). A two point calibration of the sensor using air and nitrogen-purged water was applied.

3.2.5 Integrated sampling for offline analytics

To enable sterile withdrawing of samples during continuous operation of the modified Sapromat, a sampling port was constructed. A brass
T-piece (Serto AG, Aadorf, Switzerland) was mounted between the cultivation flask and the oxygen production flask which are connected by a tube. A stainless steel cannula (VWR, Dietlikon, Switzerland) fitted with a teflon tube which immersed in the fermentation broth was inserted through the remaining opening of the T-piece. Attached to the cannula was a 360° three way valve fitted with a non-return valve (Fisher Scientific SA, Wohlen, Switzerland) on one side and a sterile filter on the other side. Through the non-return valve, samples could be withdrawn with a syringe in a monoseptical manner. To empty the sampling tube and to compensate for the pressure drop in the system after sampling a corresponding amount of air was injected through the sterile filter. In this work, the sampling system was operated manually.

3.2.6 Determination of oxygen mass transfer coefficients

The volumetric gas liquid mass transfer coefficient ($k_{L,a}$) was measured by three different methods: (i) In the dynamic gassing out method (Van’t Riet, 1979), distilled water in the measuring flasks was deoxygenated by purging with nitrogen while already applying the required stirring frequency until a constant DO signal could be defined. After stopping the N\textsubscript{2} supply and addition of ambient air in the gas room by a tube in the upper part of the flasks, the increase of DO over time was recorded. All measurements were performed at least in triplicates at a temperature of 28 °C. A correction regarding the response time of the sensor was not performed. Such a correction is necessary if the probe’s response time $\tau$ is in the same order of magnitude as the mass transfer response time of the system $1/k_{L,a}$. According to Van’t Riet, the uncorrected data do not deviate more than 6% from the corrected ones if $\tau < 1/k_{L,a}$ (Van’t Riet, 1979). Our $k_{L,a}$’s were typically on the order of 100 $1/h$, corresponding to system response times of 36 s. The probe’s response time was measured for all applied stirring rates by addition of 5 mL 1 M Na\textsubscript{2}SO\textsubscript{3} solution to 45 mL fully oxygenated water and subsequent measuring the time needed to record 63% of this step change to DO = 0%. The response time under the applied conditions amounted to 25 s, independently of the applied stirring rate. (ii) For the sulfite ox-
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oxidation method, reaction conditions were chosen as described (Hermann et al., 2003). Briefly, a 0.5 M sodium sulfite (99% purity, Fluka, Buchs, Switzerland) solution was prepared in a 12 mM NaH₂PO₄ buffer (pH 8). The sulfite solution was filled into the Sapromat reaction vessels and CoCl₂ was added to a concentration of 0.5 µmol/L. The corresponding kₜ,a value could be calculated according to equation 3.1:

\[
OTR = k_{t,a} \cdot (DO^* - DO)
\]

assuming complete oxygen depletion in the liquid and an equilibrium oxygen concentration (DO*) in the sodium sulfite solutions of 5.28 mg/L (Schumpe et al., 1978). The oxygen consumption rate was measured with the Sapromat at a temperature of 28 °C. (iii) Finally, the kₜ,a was calculated during the cultivations based on the data pair OTR and DO according to equation 3.1. The equilibrium oxygen concentrations DO* were calculated to 5.9 mg/L for M9* medium and to 6.5 mg/L for LB medium (Schumpe et al., 1982). Where the maximum oxygen production capacity of the Sapromat limited OTRs to an artificially low value, the kₜ,a values were determined in the period before the onset of this limitation.

3.2.7 Cultivations of *Escherichia coli*

All cultivations with *E. coli* were performed at 37 °C. *E. coli* LJ110 (W3110 fnr+) was used throughout this study (Zeppenfeld et al., 2000). Cultures were inoculated from precultures in identical medium to an optical density at 600 nm (OD₆₀₀) of 0.1 except for the diauxic growth experiments, where precultures were grown on M9* with 2 g/L glucose. Culture volume was 50 mL and the stirring rate was set to 750 rpm unless indicated otherwise. All experiments have been performed in triplicates to ensure the validity of the measured data.

LB medium has been described elsewhere (Sambrook and Russell, 2001). Minimal medium M9* consisted of (in g/L) Na₂HPO₄ 20.3, KH₂PO₄ 9, NaCl 0.5, NH₄Cl 1 and each 1 mL/L of 1 M MgSO₄, 0.1 M CaCl₂ and US* trace element solution (Panke et al., 1999). The high concentrations of phosphate salts were employed to buffer the medium at pH 7. The pH of
the cultivations was controlled regularly and always found to be above 6.5, so that culture profiles were hardly influenced by pH effects. M9* was supplemented with 2 s/L glucose except for diauxic growth experiments where 1 s/L glucose and 1 s/L lactose were supplied. To investigate the effect of nitrogen limitation, the amount of NH₄Cl in M9* was reduced to 0.125, 0.25 and 0.5 s/L. These values were calculated based on an assumed E. coli biomass composition of CH₁₇O₀₄₉N₀₂₄ and a biomass over substrate yield coefficient (Yₓₛ) for batch growth of E. coli on glucose of 0.38 s/g (Varma and Palsson, 1994; Xu et al., 1999). Thus, to balance the nitrogen contents, a M9*/glucose medium requires 5 g of glucose per 1 g of NH₄Cl.

3.2.8 Analytical methods

OD₆₀₀ values were measured with a BioPhotometer (Vaudaux-Eppendorf, Schoenenbuch, Switzerland). One OD₆₀₀ unit corresponds to 0.35 s/L cell dry weight as shown by accompanying cell dry weight measurements (data not shown). Glucose and lactose were quantified by HPLC on a Merck LaChrom system L-7200 (VWR, Dietlikon, Switzerland) equipped with an RI detector (Merck LaChrom L-7490) and an Ultrahydrogel 250 column (Waters Corporation, Milford Massachusetts, USA) flushed with water as eluent. The flow rate was set to 1 mL/min and HPLC runs were performed at ambient temperature. Acetate concentrations were determined with an enzymatic test kit (Soil Diagnostics GmbH, Viernheim, Germany).

3.2.9 Kinetic growth model

An unstructured kinetic model based on a Monod growth model was used to determine theoretical OTR data. Biomass formation, glucose consumption and oxygen consumption rates were described by equations 3.2 to 3.6:

\[
\frac{dcX}{dt} = \mu \cdot cX
\]
where \( r_x, r_s, \) and \( r_{O_2} \) denote the rates of formation or consumption of biomass, substrate, and oxygen, \( c \) the corresponding concentrations, \( \mu \) the specific growth rate, and \( K_S \) Monod’s saturation constant for the substrate in question (10 mg/L for glucose (Akesson et al., 1999)). Yield coefficients for biomass/glucose (0.38 g/g (Varma and Palsson, 1994)) and biomass/oxygen (0.98 g/g (Marison and von Stockar, 1986)), were taken from the literature, where the corresponding experiments had been conducted in similar conditions with respect to media composition and carbon source concentration. The end of the lag-phase was chosen as the actual starting time. Maintenance was not considered because the values from the literature were overall yield coefficients, where the maintenance contribution had already been included. The model equations were solved numerically by an ordinary differential equation solver (Berkeley Madonna, Version 7.0.1, Macey and Oster).

3.3 Results

3.3.1 Technical modifications of the Sapromat E system

The Sapromat’s current design allows a maximum oxygen production of 50 mg/h, which should roughly be sufficient oxygen for 50 mL of a typical
E. coli culture growing exponentially with a specific growth rate of 0.5 h⁻¹ up to a biomass concentration of 2 g/l (see above for coefficients). However, first experiments with the unmodified Sapromat rapidly indicated that the provided stirring rates were inadequate to produce the corresponding mass transfer coefficients (data not shown). Consequently, we implemented the possibility for increased stirring speeds and adapted stirrers and flask geometry accordingly (see section 3.2.2). Stirring rates up to 800 rpm could then be employed in a stable fashion. Furthermore, in order to confirm the OTR measurements and to ensure experimenting under oxygen unlimited conditions, we implemented optical DO sensors. The DO sensors also allowed the fast determination of the system’s kLa-values under various reaction conditions. Finally we implemented a sampling station that allowed us to withdraw samples under monoseptical conditions without disturbing the OTR measurements and without interrupting the aeration of the cultures.

3.3.2 Selection of a CO₂ adsorbent

The adaptations mentioned above still delivered maximum OTR values for standard M9* E. coli cultures that were about 40% lower than expected. Furthermore the DO and OTR signals did not show the expected inverse trend. Consequently, we investigated the carbon dioxide mass transfer. The measurement principle of the Sapromat requires the fast and complete adsorption of produced CO₂. Originally, approximately 10 g of soda lime pellets, which are placed in a glass pocket in the neck of the cultivation flask, were used for this purpose. We evaluated soda lime, lithium zirkonate Li₂ZrO₃, lithium silicate Li₄SiO₄, aqueous KOH (30%), and a hydrophobic molecular sieve as alternative CO₂ adsorbents that might be able to adsorb the CO₂ more rapidly as the soda lime. The amounts of the different adsorbents (and with them the surface area available for CO₂ adsorption) were increased for cultivations of E. coli in M9* until the obtained OTR profile did not change anymore, the expected OTRmax values were reached (see below), and the expected exact inverse behavior of DO and OTR was observed. This situation was reached with 15 g of Li₂ZrO₃ and Li₄SiO₄ and 25 g of soda lime, while this was not possible with the other adsorbents (data not
shown). The soda lime showed a pronounced water adsorption activity, which made it less suitable for our purposes. Li$_2$ZrO$_3$ could be easily regenerated by desorption of CO$_2$ at elevated temperature (Nakagawa and Ohashi, 1998). Accordingly, we performed subsequent experiments with 20 g of Li$_2$ZrO$_3$ per flask as CO$_2$ adsorbent. With this amount, we could maintain strictly inverse behavior of DO and OTR curve under all the investigated culture conditions up to the maximum oxygen production capacity of the device, which confirmed that the adaptations of the Sapromat allowed sufficiently rapid removal of CO$_2$.

### 3.3.3 Growth of *E. coli* on glucose minimal medium - overflow metabolism

In order to prove the suitability of the respirometric measurement principle after the adaptation of the device and study its potential for detecting a number of important process development-related cultivation phenomena, we investigated a culture that could be easily balanced such as growth of *E. coli* on a mineral medium with glucose (Fig. 3.2). We compared predicted and actual OTRs for the growth of *E. coli* in 50 mL of defined M9* medium with glucose in batch mode and determined the maximum specific growth rate from the DO signal. This value was then used as a parameter in an unstructured growth model employing yield coefficients from the literature and we compared the resulting predictions for the oxygen consumption rate with the experimental data (Fig. 3.2).
Figure 3.2: Growth of *E. coli* cells on defined minimal medium with 2 g/l glucose. a) Comparison of simulated and measured data for OTR and DO. The measured OTR curve is started plotting after occurrence of the first oxygen production cycle, which does not correspond to the culture’s lag time because the first 3 production cycles are missing due to water vapor related effects in the system (see section 3.2). b) Cell dry weight development expressed as OD_{600} (measured and simulated data) and acetate formation. The last data points for acetate are not connected in order not to suggest an obviously unrealistic course of acetate in time.
As the reutilization of acetate was not implemented into the model, only the first growth phase on glucose served as the basis for comparison. Simulated and measured data showed excellent agreement for OD₆₀₀, DO and OTR for the period between 2 and 6h (Fig. 3.2 a), corresponding to the period of growth on glucose. After 6h, a brief second growth phase was observed, which can easily be explained by growth on acetate which had been produced before by overflow metabolism (Fig. 3.2 b). The OTR reached a maximum value of 0.49 s/L after 6h. Concomitantly, the maximum acetate concentration of 0.23 s/L was reached, which dropped subsequently due to acetate consumption. The period of acetate consumption could be accurately recorded from the DO and the OTR measurements.

### 3.3.4 Volumetric oxygen mass transfer coefficients without cultivation

In order to characterize the system further, we investigated the volumetric oxygen mass transfer coefficients of the adapted Sapromat system. The kₐLa depends principally on the power input into the culture and the aeration rate. The former is dependent on the stirring rate, which in turn might also influence the latter by changing the aeration mode from surface aeration only to additional aeration by bubble generation. Furthermore, the geometry of the flask might influence the available surface to exchange oxygen, so the kₐLa-values were determined for increasing stirring rates and different liquid volumes. Fig. 3.3 shows the results of the kₐLa measurements for different filling volumes and stirring rates measured with two different methods, the dynamic gassing out method and the sulfite oxidation method. Enhancing the stirring rate from 300 to 750 rpm resulted in a 3.5-fold higher kₐLa value for reaction volumes of 25 and 50 mL as determined by the dynamic gassing out method. The kₐLa values at stirring speeds of 300 and 750 rpm were identical for filling volumes of 25 or 50 mL, whereas at stirring speeds of 450 and 600 rpm higher kₐLa’s were reached for the 25 mL flasks. The maximum kₐLa that could be reached for these two culture volumes amounted to 100 1/h. This corresponds to a maximum OTR of 0.65 s/Lh and a total oxygen production of 32.5 mg/h for the 50 mL culture, which is lower than
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Figure 3.3: Volumetric oxygen mass transfer coefficients: $k_{L}a$-values measured by different methods in 500mL Erlenmeyer flasks with varying filling volumes and stirring rates: The dynamic gassing out method (DGOM) was used for volumes of 25 and 50mL of water and the sulfite oxidation method (SOM) for volumes of 37 mL of sulfite solution. Furthermore $k_{L}a$'s were calculated from DO- and OTR data pairs during different *E. coli* cultivations on LB or M9* with glucose.

The Sapromat's maximum oxygen production capacity. However, as stirring became unreliable beyond a stirring rate of 800 rpm, this defined the upper limit for the $k_{L}a$. Qualitatively similar results were obtained with the sulfite oxidation method, although much higher $k_{L}a$'s of up to $225 \, l/h$ were measured. An increase of the stirrer speed from 400 to 800 rpm resulted also in a $k_{L}a$ enhancement by a factor of 3.5.
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Figure 3.4: Growth of E. coli on LB medium in different culture volumes in 500mL flasks. Shown are OTR (circles) and DO (line) profiles. b) shows the reproducibility of two DO-curves and three OTR curves, respectively.

3.3.5 Volumetric oxygen mass transfer coefficients with cultivation: Growth of E. coli on LB medium

As the k_{La} values can vary significantly between the situation with and without cultivation, we also wanted to determine k_{La} values with a biological test system. We selected E. coli as a typical biotechnologically relevant strain with high growth rates especially in complex media and calculated the volumetric oxygen transfer coefficients for growth on LB medium from the measured OTR and DO data (Fig. 3.3). The determined values were between the values determined by gassing out and sulfite oxidation and ranged from 160 \text{1/h} for a cultivation volume of 50mL to 80 \text{1/h} for a cultivation volume of 100mL. These k_{La}-values already indicated a potential for oxygen limitation for growth of E. coli on LB medium for cultivation volumes larger than 50mL. Indeed did the DO measurements confirm oxygen unlimited growth of cells in 50mL
medium, with a minimum DO of 1.3 mg/L (equivalent to 20% relative to air saturated medium). DO was depleted for 3 min in the 75 mL cultures and for 1.1 h in the 100 mL culture. Correspondingly, we found similar maximum OTR values of 0.9 g/Lh for cultivation volumes of 25 and 50 mL, but plateaus in the OTR profiles for cultivation volumes of 75 and 100 mL (Fig. 3.4). These plateaus marked the maximum oxygen production capacity of the Sapromat of 50 mg/h, limiting OTR\text{max}\text{-values} to 0.67 g/Lh (75 mL) and 0.5 g/Lh (100 mL) for these cultivations. Based on these results, we set the standard cultivation volume to 50 mL.

### 3.3.6 Glucose-lactose diauxic growth

![Graph showing diauxic growth of E. coli on glucose and lactose. DO and OTR were recorded online, OD\text{\textsubscript{600}}, glucose, and lactose were measured offline.](image)

**Figure 3.5:** Diauxic growth of *E. coli* on glucose and lactose. DO and OTR were recorded online, OD\text{\textsubscript{600}}, glucose and lactose were measured offline.

Next, we investigated the suitability of the Sapromat-system to analyze diauxic growth situations as they are common in complex media with multiple C-sources. Therefore, we cultivated *E. coli* in defined medium with two carbon sources, glucose and lactose (Fig. 3.5). As expected,
3. MEDIUM-THROUGHPUT OTR MEASUREMENTS

Glucose was consumed first and was depleted after 4.5 h, as indicated by an increase in DO signal, the decrease of OTR, and the offline glucose measurement. Apparently, the transition from glucose to lactose metabolism was very fast: the DO signal rose again after only 4 min. This conversion could also be detected by measurements of optical density, however frequent sampling was required. After an additional 2 h, lactose was consumed as well, again indicated by the antagonistic behaviour of DO and OTR and by the offline lactose measurements. A short third growth phase ensued, which is assumed to be due to acetate consumption. In summary, diauxic growth could be easily analyzed with the oxygen related signals, and the OTR measurements were sensitive enough to detect the behaviour even though the transition from one carbon source to the next was rather quick.

3.3.7 Nitrogen limited growth of *E. coli*

Another frequently encountered situation in process development is medium optimization and the corresponding detection of limitation in specific nutrients. We investigated depletion in ammonium as the nitrogen source. Therefore, we provided minimal media with 2 g/L glucose and three different concentrations of NH₄Cl: 0.5 g/L, 0.25 g/L and 0.125 g/L. Only the highest concentration should be sufficient to have the growth limited by the carbon source rather than the nitrogen source.

The effect of nitrogen limitation could be observed easily from the OD₆₀₀ and glucose data (Fig. 3.6 a). The nitrogen unlimited culture consumed all glucose in 6.5 h and grew exponentially until a maximum OD₆₀₀ of 2. When the concentration of the nitrogen source was reduced to a value of 0.25 g/L NH₄Cl, exponential growth ended after 5.8 h at an OD₆₀₀ of 1.3 and consumption of 1.1 g/L glucose. A further reduction of the NH₄Cl concentration to 0.125 g/L led to an OD₆₀₀ of 0.7 and a glucose consumption of 0.6 g/L at the onset of nitrogen starvation. Based on the available yield coefficients and biomass composition, a medium containing 0.125 g/L of NH₄Cl should allow the consumption of 1.25 g/L of glucose to form 0.48 g/L of biomass, corresponding to an OD₆₀₀ of 1.4. Consequently, the measured and expected values were in
good agreement, also for a NH$_4$Cl concentration of 0.125 $\text{g}\text{/L}$. The OTR and DO data gave a more detailed picture of the growth behaviour of the cells under nitrogen limitation (Fig. 3.6 b and c). The nitrogen unlimited culture grew in two phases: a) unlimited growth on glucose until depletion after 6 h; b) unlimited growth on acetate produced by the culture itself until 8 h. In the nitrogen-limited culture with 0.25 $\text{g}\text{/L}$ of NH$_4$Cl, three distinct phases could be detected: a) unlimited growth on glucose (0 to 5.7 h); b) maintenance on glucose until glucose depletion (5.7 to 8.5 h) and c) maintenance on acetate (8.5 to 11.7 h). As in both maintenance phases the DO continued to drop after the increase, we assume that residual growth continued either on some N-containing compounds that have reached the medium in the growth phase or because the cells turned to intracellular sources of nitrogen, as it is well established for E. coli (Gyaneshwar et al., 2005). When the nitrogen content of the medium was reduced further to 0.125 $\text{g}\text{/L}$ NH$_4$Cl, the same three phases could be observed; phase a) 0 to 4.8 h; phase b) 4.8 to 17.9 h, and phase c) 17.9 to 30.5 h (data shown only for the first 20 h). In summary, the Sapromat-based device is capable of revealing rather subtle changes in culture physiology, either by OTR- or DO-measurement.

3.4 Discussion

3.4.1 Scope of the OTR measurement principle

Modern bioprocess development strives for increased data densities and control of a more comprehensive parameter set at ever smaller cultivation scales. One essential parameter here that needs to be carefully controlled is the oxygen concentration in the medium (Buchs, 2001; Zimmermann et al., 2006). The amount of available oxygen may alter growth rates (Arras et al., 1998; Ensari and Lim, 2003), product formation in general (Freyer et al., 2004), recombinant protein overexpression (Bhattacharyya and Dubey, 1997; Garcia-Arrazola et al., 2005), secondary metabolite formation (Clark et al., 1995; Frykman et al., 2002; Vardar and Lilly, 1982), by-product formation like acetate (Martins and Tempest, 1991;
Figure 3.6: Nitrogen limitation in *E. coli* cultivations. a) Glucose and OD$_{600}$ values for growth of *E. coli* in various nitrogen limited and unlimited media. 
b) OTR and c) DO data of nitrogen limited and unlimited *E. coli* cultivations. The inlets show a magnification of the DO and OTR curve for the culture with 0.125 g/L of NH$_4$Cl.
Phue and Shiloach, 2005), and even fungal morphology (Papagianni, 2004). Consequently, DO and OTR data are key parameters to evaluate the proper course of the cultivation.

At the same time, the OTR can be used as a valuable additional process analytic tool: metabolic activity of non-growing cultures, growth kinetics, substrate or product inhibitory effects can be studied on the basis of OTR measurements. Finally, by providing data on the oxygen requirements of the culture, OTR measurements can provide essential information for scale up.

The OTR measurement principle applied in this work is based on the fact that the oxygen-consuming respiratory activity of cells leads to a detectable pressure drop in a closed system. This requires that any gaseous products of cell metabolism are removed instantaneously from the gas phase. The primary gaseous product of aerobically growing cells is carbon dioxide, which is why a CO₂ adsorption cell is implemented into the system. To be able to measure correct oxygen transfer rates, the virtually complete adsorption of CO₂ has to be as fast as the oxygen consumption of the cells, otherwise too low OTRs would be measured. After optimizing the CO₂ adsorption step, reliable OTRs could be measured with the Sapromat even at elevated oxygen consumption rates. However, the virtually complete removal of CO₂ from the gas phase rises the point that these conditions are non-physiological, because CO₂ also serves as a substrate in mineral medium during growth (Onken and Liefke, 1989; Zanzotto et al., 2004). Therefore, we let E. coli grow on M9*/glucose in the adapted Sapromat in flasks operating in respirometric mode (stirred, electric oxygen generation) and in stirred flasks stoppered with cotton-plugs, and compared the growth behavior to a culture growing in a shake-flask. The cultures showed no difference in growth behavior. Apparently, the liquid phase in the respirometric mode serves to a sufficient extent as a delay element in eliminating the CO₂ from the system.

It is also clear that the measurement principle will fail if gaseous products or products with a high vapor pressure (such as ethanol) are formed because they will reduce the pressure difference. Furthermore, the closed nature of the system will be disadvantageous in such situations as it does not allow stripping of volatile compounds. Consequently, care has to be taken when validating the system for strains that are known to excrete
volatile compounds under the selected growth conditions.

3.4.2 Oxygen transfer capacity

The Sapromat E had originally been developed for environmental investigations, such as the examination of the biodegradability of certain compounds or the determination of the load of organic pollutants in a waste water sample (Bouchez et al., 1995; Martius et al., 1996; Reuschenbach et al., 2003; Ros et al., 1990; Verstrae et al., 1974). The oxygen consumption rates during these kinds of experiments are low, typically in the order of 0.75 g/L over 10 days (Martius et al., 1996). This requirement for small oxygen transfer rates was reflected in the original design of the Sapromat. Consequently, we re-equipped the device to sustain high stirring speeds and then characterized the oxygen transfer capacity by the dynamic gassing out method, sulfite oxidation method, and from cultivations (Fig. 3.3). The differences between these methods have been documented and explained in the literature. In the sulfite oxidation method, the physical mass transfer can be enhanced if a considerable part of the reaction takes place in the gas liquid boundary layer. This is generally the case if the Hatta number is greater than 0.3 (Linek and Vacek, 1981; Maier et al., 2001), which can already be the case even if no catalyst at all was added to the sulfite solution, depending on the concentration of trace impurities and the actual value of the gas liquid mass transfer coefficient (Linek and Benes, 1978). Unfortunately, we could not determine the Hatta number as we did not determine the actual rate constant of oxygen consumption in the bulk liquid (Maier et al., 2001). Therefore, we cannot exclude that the $k_{La}$ is overestimated because of a too fast reaction. Furthermore, coalescence inhibition due to the dissolved sulfite tends to lead to an overestimation of the $k_{La}$'s (Van't Riet, 1979). On the other hand, the dynamic gassing out tends to underestimate mass transfer coefficients due to the non-instantaneous change from nitrogen to air (Dang et al., 1977; Linek et al., 1987). Furthermore the probe's response time is in the same order of magnitude as the mass transfer response time of the system, which also leads to slightly lowered $k_{La}$ values (Van't Riet, 1979). Measurements of $k_{La}$ values based on DO and OTR-data usually best reflect the
true situation in the culture. However, no quantitative relations exist between the three methods, which makes quantitative comparison across methods difficult. Consequently, we selected all the above mentioned, most frequently used methods to produce a broadly applicable data set. Measurement of the $k_{L\alpha}$ by the sulfite oxidation method resulted in the highest measured values of up to $225 \frac{1}{h}$. Measurement by dynamic gassing out indicated values of up to $100 \frac{1}{h}$. The relative increase in $k_{L\alpha}$ from increasing the stirrer speed was similar with both methods. DO- and OTR-based $k_{L\alpha}$-measurements resulted in again slightly different values from $130$ to $160 \frac{1}{h}$ in $50 \text{mL}$ fermentations stirred with $750 \text{rpm}$, corresponding to maximum possible OTRs of up to $1 \frac{s}{L \cdot h}$. These data were in between the $k_{L\alpha}$ values obtained with the other two methods. The maximum possible cultivation volume for an experiment in the adapted Sapromat needs to be re-considered for each experiment in the light of the maximum oxygen production rate of the device, the cell dry weight that can be obtained from the medium, the maximum specific growth rate of the selected strain in the specific medium, and this strain’s yield coefficient for biomass over oxygen. Each of these parameters can considerably change the maximum possible volume that can be applied without risking oxygen depletion. As a rule of thumb, with the oxygen transfer rates confirmed in this study it is possible to run LB-supported $E. coli$ cultivations of $50 \text{mL}$ at DO levels always higher than $20\%$.

### 3.4.3 Biological validation and applications of the measuring system

In order to show the suitability and the potential of the online OTR measurement system of the Sapromat several $E. coli$ experiments were carried out, where besides the OTR also DO, $\text{OD}_{600}$ and concentrations of carbon sources and metabolic byproducts were measured. The results compared well with simulations based on a kinetic growth model, that typically describes the growth of bacterial cells without major metabolite production well (Nielsen et al., 2003) (Fig. 3.2), confirming the suitability of the respirometric measurement system in the adapted Sapromat setting. The short adaptation time in the transition from glucose to acetate
consumption is in agreement with the literature (Ishikawa et al., 1981). Small changes in growth rate on complex media could also be resolved by DO and OTR-measurements (Fig. 3.4) and the expected growth behavior of E. coli cultures for a glucose-lactose diauxie (Epstein et al., 1966; Fischer et al., 1998; Loomis and Magasani, 1967) could be reproduced satisfactorily, even though we observed a very short transition time of only 4 min, which is in contrast to some earlier reports (Epstein et al., 1966; Fischer et al., 1998), but agrees with transition times from biomass measurements with strain LJ110 (Bettenbrock et al., 2006). Finally, the exact time point of nitrogen depletion could be determined in nitrogen-limited cultures and a detailed picture of the subsequent behaviour of the strain could be obtained (Fig. 3.6). In summary, the adapted Sapromat was perfectly capable to detect and accurately measure a number of typical process development-related phenomena in a parallelizable format.

3.4.4 Evaluation of the adapted Sapromat

The development of controllable and parallelized small scale bioreactors in the volume range from below 1 to 500 mL has become a crucial step in a modern accelerated process development trajectory (Fernandes and Cabral, 2006; Kumar et al., 2004). At the intermediate or shake-flask scale, a number of systems have become recently available that try to improve and accelerate two of the typical steps in bioprocess development, both of which are fundamental to a rapid development:

(i) Systems that implement performance characteristics that are similar to (or even better than) those of laboratory-scale bioreactors, including careful process control such as DO and pH maintenance and allowing for high cell densities (Doig et al., 2005b; Frachon et al., 2006; Ge et al., 2006; Puskeiler et al., 2005; Tang et al., 2006) and

(ii) systems that increase the data density in shake flask experiments while maintaining the characteristics typical for screening, such as diluted media that are compatible with relatively small oxygen transfer rates, simple agitation mechanisms such as shaking, and batch protocols (Anderlei and Buchs, 2001; Anderlei et al., 2004). As discussed above, the crucial process parameters to measure for the latter group, to
which the adapted Sparomat would belong, are the aeration status and the oxygen transfer rate. Here, the adapted Sapromat system should make a valuable contribution to an accelerated bioprocess development scheme. The achievable volumetric oxygen mass transfer coefficient of 160 1/h compares well to those of shaken Erlenmeyer flasks, if common standard conditions are chosen and the same filling volume is applied. A 500 mL Erlenmeyer flask filled with 50 mL and shaken with a diameter of 50 mm at 250 rpm exhibits a $k_L a$ value of 68 1/h, whereas at a filling volume of 20 mL a value of 220 1/h is reached (Maier, 2002; Maier et al., 2004). These values should also be high enough to eliminate in many instances the requirement to switch to baffled flasks, which typically allow higher $k_L a$-values (Maier, 2002), but might interfere with reproducibility (Buchs, 2001). The implemented online DO measurements enabled the detection of small and quick changes in the cell metabolism (Fig. 3.3).

Furthermore, the Sapromat has been successfully applied for over three decades and more than 1000 devices have been sold (H+P Labortechnik, personal communication). In our version, it is 12-fold parallelized, but can be easily upgraded to 48 fold. As the device is a non-moving platform, sampling without interrupting the aeration by stirring and without disturbing the OTR measurements is possible. This can be important, because even a short interruption of aeration can lead to oxygen starvation of the cells with unwanted effects, e.g. the expression of stress related genes (Sanden et al., 2003; Wittmann et al., 2003).

We conclude that several applications of the device are possible especially in the early stages of bioprocess development during screening and first steps of strain characterisation. The device is well suited e.g. to perform medium optimization, to standardize precultures, to study product/educt inhibition or to investigate the growth behavior of different mutants.

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Chapter 4

High concentrations of clavulanic acid decreases glycerol consumption and oxygen transfer rates in cultures of *Streptomyces clavuligerus*

Abstract

Product inhibition of biological production systems is an important and widely observed phenomenon with prominent implications for the design and ultimately the success of biotechnological processes. We investigated whether such effects limited the maximal titers in the production of the $\beta$-lactamase inhibitor clavulanic acid (CA) in *S. clavuligerus* cultivations. First, we implemented a medium scale (50 mL) cultivation device...
enabling the parallel measurement of the oxygen transfer rate (OTR) of *S. clavuligerus* cultures. Comparable results to a laboratory scale aerated stirred tank reactor could be achieved after optimizing the stirring system, which in is optimized form enabled $k_La$ values of up to 192 $l/h$ during cultivations, thus allowing oxygen unlimited growth in a production medium. With this, we investigated the effect of CA additions to the cultures and supported the OTR measurements with sampling for glycerol consumption, the main carbon source, and CA production. Increased levels of CA severely interfered with the physiology of the producing strain at least at and above 1.5 $g/L$ CA, as indicated by a dose depended decrease in maximal OTRs and glycerol consumption rates. However, inhibition of CA synthesis at the level of the current maximum wild-type titer of 0.4 $g/L$ could not be observed, as CA still continued to accumulate in the medium after addition of CA in this concentration range.

### 4.1 Introduction

Clavulanic acid (CA) is a potent inhibitor of $\beta$-lactamases produced by some strains of pathogenic microorganism resistant to $\beta$-lactam antibiotics thereby enabling efficient treatment of infectious diseases (Reading and Cole, 1977). It is one of the active ingredients in Augmentin, a commercially highly successful antibiotic medicine. The wild-type producing strain *Streptomyces clavuligerus* typically yields between 0.1 and 1 $g/L$ of the compound in cultivations employing usually complex medium with glycerol or fatty acids as main carbon source and soy bean derivatives as nitrogen source (Baptista-Neto et al., 2000; Chen et al., 2003; Gouveia et al., 1999, 2001; Large et al., 1998; Mayer and Deckwer, 1996). Further increase of the possible final concentration is desirable to increase process economy. However, it is not clear what effect a further increase in the CA titer will have on the physiology of the producing strain and on the decomposition of the product. Remarkably, fed-batch fermentations with glycerol as fed carbon source lead to a maximum CA titer of approximately 0.4 $g/L$ long before the glycerol consumption stopped. One possible reason for this behavior is the well known CA decomposi-
tion in the fermentation broth (Mayer and Deckwer, 1996; Roubos et al., 2002). Alternatively, product inhibitory effects of CA might be responsible, which are often found in antibiotic fermentations (Demain, 1974). Product inhibition might occur on the level of the producing enzymes when the synthesis pathway is turned off by accumulating product and the strain physiology remains essentially unaffected, as it is the case e.g. for cycloheximide (Dykstra and Wang, 1990), nisin (Pongtharangku and Demirci, 2007) and aurodox (Liu et al., 1977). Alternatively, the product can interfere with other cellular functions which typically lead to interference with the producer’s physiology, frequently reflected in decreased carbon source consumption rates (Nyiri et al., 1963), a prolonged lag phase (Malik and Vining, 1970), growth inhibition (Castaldo et al., 1996; Jia et al., 2006) or induction of cell lysis (Schmitt and Freese, 1968). Specific resistance mechanisms might increase the product threshold concentration for this behavior, but do not change this behavior fundamentally.

In this work, we investigated the effect of elevated CA concentrations on its producer strain *S. clavuligerus* in detail, specifically the effect on carbon source consumption. Previous work already indicated, that the addition of 1.3 s/l CA led to a decrease of the specific growth rate from 0.18 h⁻¹ for standard fermentations to 0.11 h⁻¹ (Roubos et al., 2002), suggesting an impact of increased CA concentrations on strain physiology. As any future work directed at either removing CA actively from the medium or at improving the resistance of *S. clavuligerus* will undoubtedly benefit from experimental parallelization and parallelized cultivation platforms equipped with on-line oxygen transfer rate measurement capability that have recently become available (Anderlei et al., 2004; Brethauer et al., 2007), we also investigated whether oxygen consumption might be a similarly informative parameter for product inhibition as carbon source consumption.
4.2 Materials and methods

4.2.1 Parallel OTR and DO measurements with modified Sapromat E

OTR and DO measurements were performed with a modified version of the Sapromat E (H+P Labortechnik, Munich, Germany). A detailed description of the measurement principle and the modifications can be found elsewhere (see chapter 3). Briefly, it consists of 12 culture flasks, which are either magnetically stirred or shaken in an external incubator. Each flask is connected to a carbon dioxide trap containing Li$_2$ZrO$_3$, a switch manometer and an electrochemical oxygen production unit in a closed system. The oxygen partial pressure decreases upon oxygen consumption for respiration or growth, this pressure drop is detected by the differential manometer and compensated by electrochemically produced oxygen, which is the parameter that is actually measured. The carbon dioxide that is produced from respiration and that would interfere with the measurement is captured in the CO$_2$ trap. Cumulated oxygen production is recorded and further processed to OTR values.

Dissolved oxygen levels were measured with an optical system (Presens GmbH, Regensburg, Germany) by dynamic quenching of luminescence by O$_2$ in a sensor spot mounted inside the culture flask as described elsewhere (Brethauer et al., 2007). The signal was read out via a polymethylmethacrylate optical fiber. A two point calibration of the sensor using air and nitrogen-purged medium was applied.

4.2.2 Magnetic stirrer bars for aeration of cultures

The stirring system of the Sapromat is dimensioned for stirrer bars with a magnetic pole distance of approximately 40 mm. Commercially available longer stirrer bars can not be employed because the magnetic interaction is too weak to enable stable stirring even at low stirrer speeds. Next to the standard stirrer (stirrer 1: a cylindrical magnetic stirrer bar, 8 mm diameter, 40 mm length, samarium cobalt core (Semadeni, Ostermundingen, Switzerland)), we therefore used a custom-designed extended stirrer:
Stirrer 2 was constructed from six cubic NeFeB magnets with an edge length of 5 mm (Webcraft GmbH, Uster, Switzerland) that were placed in a 65 mm long Teflon tube. Both ends of the tube were sealed with silicone (Elastosil E41, Wacker-Chemie GmbH, Munich, Germany) and a second Teflon tube of 10 mm length was placed around the stirrer in its center to minimize the surface contact of stirrer and bottom and to improve stirring stability. This stirrer type featured a high stability at stirring rates of up to 1000 rpm.

### 4.2.3 Determination of oxygen mass transfer coefficients

The volumetric gas liquid mass transfer coefficient ($k_L a$) was measured as described recently with the dynamic gassing out method and in fermentation broths by measuring OTR and DO concomitantly (see chapter 3). The response time of the oxygen sensor was measured to be 20 s in case of stirrer 1 and 6 s for stirrer 2, respectively, therefore a correction of the measured $k_L a$ values was not necessary. The equilibrium oxygen concentrations $DO^*$ were calculated to 6.5 mg/L for the employed production medium (see below) (Schumpe et al., 1982).

### 4.2.4 Cultivations of *S. clavuligerus*

Precultures of *S. clavuligerus* DSM 738 (DSMZ, Braunschweig, Germany) were produced from an aliquot of 100 μL of a spore suspension from a deep frozen (−20°C) stock that was inoculated into 20 mL ISP1 medium (Mayer and Deckwer, 1996) and incubated at 30°C on an orbital shaker at 200 rpm for 24 h. Next, 47.5 mL of production medium (glycerol 20 g/L, soybean extract 200 mL/L, peptone 5 g/L, 3-(N-morpholino)propanesulfonic acid buffer 100 mM, antifoam 0.2 mL (Industrol DF204, BASF, Ludwigshafen, Germany), adjusted to pH 7 with potassium hydroxide) in a 500 mL one-fold baffled Erlenmeyer flask was inoculated with 2.5 mL of the ISP1 preculture and grown for 24 h at 30°C. Soybean extract was obtained by autoclaving a suspension of
60 g/L soybean-flour in water and recovering the supernatant after centrifugation (Mayer and Deckwer, 1996).

All subsequent main cultures as described below were inoculated with 5% of this second culture and carried out at 30 °C. They were carried out either in a stirred tank fermenter, stirred Erlenmeyer flasks, or shaken Erlenmeyer flasks. The fermentation was carried out on 1.5 L scale in batch mode in a 2 L Biostat A bioreactor system (Sartorius BBI Systems GmbH, Melsungen, Germany) (Makart et al., 2007). Production medium as described above but without buffer salts was employed. Instead the pH was maintained at 7 by automated addition of 1 M NaOH or 1 M H2SO4, respectively. The minimal DO was set to 40% of air saturation and maintained by increasing the stirrer speed from a starting value of 350 rpm. The aeration rate was set to 1.5 L/min. CO2 and oxygen in the exhaust gas were measured by infrared gas analysators (MBE Electronic AG, Schwerzenbach, Switzerland) from which the OTR was calculated by a gas balance.

Shaken cultures were operated at a volume of 25 mL in 500 mL Erlenmeyer flasks with four baffles shaken at 200 rpm and 50 mm amplitude. The flasks were connected via 1.5 m of PVC tubing to the Sapromat system.

Alternatively, cultures of 50 mL in 500 mL unbaffled Erlenmeyer flasks were connected to the Sapromat system and stirred at 750 rpm with stirrer 1 or 650 rpm with stirrer 2, respectively.

Cultures were carried out in duplicates, with additional parallel runs without the connection to the Sapromat system in order to sample for CA and glycerol concentrations.

4.2.5 Influence of clavulanic acid on S. clavuligerus cultures

A stock solution containing 60 g/L of potassium clavulanate (International Laboratories, San Bruno, USA) in production medium was sterile filtered. Different amounts of this stock solution were added to the cultures at different time points as indicated in the results section. Cultures for negative controls were supplemented with potassium chloride to a concentration of 25 mM. This corresponds to the ionic strength of 5 g/L.
4.2.6 Analytical methods

OTR measurements were performed with the Sapromat system or via the gas balance for the fermentation (see above). Glycerol was quantified by HPLC on a Merck LaChrom system L-7200 (VWR, Dietlikon, Switzerland) equipped with an RI detector (Merck LaChrom L-7490), a diode array detector (Merck LaChrom L-7455) and an Ultrahydrogel 250 column (Waters Corporation, Milford Massachusetts, USA) flushed with water as the eluent. The flow rate was set to 1 mL/min and HPLC runs were performed at ambient temperature. CA was quantified by HPLC employing a Prontosil Eurobond C18 5.0 μm column (Bischoff Chromatography, Leonberg, Germany) as stationary phase. As mobile phase served a mixture of 30% methanol and 70% 50 mM KH₂PO₄ (pH 4.5). UV-detection at 312 nm was used to record elution volume. Prior to analysis the supernatant of a sample was mixed with an imidazole reagent (206 g/l imidazole in water, adjusted to pH 6.8 with 5 N HCl) in a volume ratio of 4:1 (Foulstone and Reading, 1982). CDW was determined by removing aliquots of 1.5 mL and centrifuging, washing and drying the biomass for at least 24 h at 95°C. CDW measurements were performed in triplicates.

4.3 Results

4.3.1 Adaptation of the Sapromat E for OTR measurements of S. clavuligerus cultivations

Product inhibition is primarily of biotechnological interest, as it limits the possible final concentration that can be achieved in a production process. Consequently, the phenomenon should be investigated under cultivation conditions that are similar to those encountered in a production process with respect to for example medium composition and dissolved oxygen concentration. As the compound whose effect needs to
be investigated is frequently rather costly, this requires the implementation of scaled-down systems that nevertheless can sufficiently imitate the optimized cultivation conditions in fermenters. To this end, we have recently implemented a parallelized cultivation platform for cultures up to approximately 50 mL that allows on-line determination of dissolved oxygen tension (DO) and oxygen transfer rate (OTR) and enables oxygen-unlimited growth of rapidly growing microbial cultures (Brethauer et al., 2007). We investigated first whether this platform and the previously established standard conditions could also be used for the reproduction of fermenter-like cultivation conditions with S. clavuligerus cultures growing in production media.

As becomes clear from the observations summarized in Fig. 4.1, the conditions (stirrer 1 operated at 750 rpm) established previously for Escherichia coli growing in diluted minimal and complex media (Brethauer et al., 2007) are not sufficient to support the growth of S. clavuligerus on production medium in 50 mL cultures without oxygen limitation for extended periods of time (Fig. 4.1 a). The DO-value dropped to 0% already after 12 h and remained at this level until the glycerol was depleted after 72 h. Correspondingly, the OTR profile remained approximately constant at 0.32 s/Lh. The culture showed a low final CA titer of 0.05 s/L, presumably due to oxygen limitation, which is much lower than previous fermenter experiments with the same medium had suggested (data not shown). The cell dry weight reached a final value of 10.5 s/L. Reducing the filling volume of the flask to 25 mL did not lead to remarkable improvements - at this volume DO measurements are difficult, but OTR, glycerol and CA measurements indicated, that these conditions were still not sufficient to support maximal bacterial activity (data not shown).

In order to improve the gas-liquid mass transfer capacity and thus delay or even totally avoid the onset of oxygen limitation, we employed longer stirrer bars and thus increased the tip speed at identical stirring frequency and thereby the volumetric power input. For a first comparison of the power inputs, we measured the gas liquid mass transfer coefficients (\(k_{\text{La}}\)) by the dynamic gassing out method in flasks filled with 50 mL of water (Fig. 4.1). Increasing the power input in this way allowed to increase the \(k_{\text{La}}\) value at a stirring rate of 750 rpm from 100 \(1/h\) to 240 \(1/h\), thus enabling a roughly 2.5-fold increase in oxygen transfer. These re-
4.3. RESULTS

Figure 4.1: a) Cultivation of *S. clavuligerus* under previously reported conditions (50 mL, 750 rpm, stirrer 1). b) *K*_<sub>L,a</sub> values determined by the dynamic gassing out method for the two different stirrers applied in water filled 50 mL flasks at 28 °C.

Results were confirmed in subsequent cultivations of *S. clavuligerus* in complex medium and using the simultaneously provided DO values together with the OTR values (Fig. 4.2) to calculate the *K*_<sub>L,a</sub> values (Tab. 4.1). Filamentous growth was the dominating growth form under both power
input regimes, as could be judged from microscopic analysis (data not shown).

<table>
<thead>
<tr>
<th>vessel</th>
<th>aeration mode</th>
<th>filling volume</th>
<th>(k_{La})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2L stirred tank reactor</td>
<td>Rushton impeller, 1000 rpm, 1vvm aeration</td>
<td>1.5</td>
<td>230</td>
</tr>
<tr>
<td>500 mL Erlenmeyer</td>
<td>stirrer 1 (standard power input), 750 rpm</td>
<td>0.05</td>
<td>50</td>
</tr>
<tr>
<td>500 mL Erlenmeyer</td>
<td>stirrer 2 (increased power input), 650 rpm</td>
<td>0.05</td>
<td>192</td>
</tr>
<tr>
<td>500 mL Erlenmeyer</td>
<td>shaken</td>
<td>0.025</td>
<td>158</td>
</tr>
<tr>
<td>with 4 baffles</td>
<td>shaken</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3.2 Similarity of different cultivation formats

Next, we compared the behavior of cultures of *S. clavuligerus* in an aerated stirred tank reactor with small scale cultures aerated either by shaking or by stirring under a high power input regime. Aeration by shaking was included in this comparison as it is a standard routine in many laboratories and the different power inputs and power input modes might lead to different behaviors.

The stirred tank culture of *S. clavuligerus* was characterized by a biphasic growth behavior with two maxima in the OTR curve (Fig. 4.2 a, b). In the first phase only little glycerol was consumed and CA production was negligible. The OTR reached a local maximum of \(0.72 \text{g} / \text{Lh}\). In the
second growth phase glycerol was consumed and CA was produced up to a maximum concentration of 0.4 g/l, which was reached after 45 hours concomitantly with glycerol depletion. The OTR in this phase reached a maximum value of 0.9 g/Lh, the maximum biomass concentration a value of 10 g/l. It should be noted, that the DO in this cultivation was maintained above 40% by adapting the stirrer speed. Remarkably, the corresponding data for cultivation in the shaken flask (Fig. 4.2 c, d) and the stirred flask (Fig. 4.2 e, f) were very similar. The biphasic pattern in the OTR curve was exhibited in these cultures as well, and the OTR values of the two local maxima were similar in both cases, though lower than in the stirred tank fermenter. The same was true for the final CA concentrations: values for the small scale cultures were similar and only slightly lower than for the stirred tank fermenter. The cell dry weight was in all cases similar. In summary, the three types of cultivation appeared to be sufficiently similar to substitute the stirred tank reactor by
the smaller cultivation vessels for the subsequent investigations.

4.3.3 Influence of clavulanic acid on \textit{S. clavuligerus} cultures

In order to investigate the effect of CA on the growth behavior of \textit{S. clavuligerus}, we added different amounts of potassium clavulanate at two different time points to the cultures and recorded the time course of CA and glycerol concentration and OTR. To exclude effects that are related rather to changes in ionic strength of the medium than to an inhibitory effect of CA on the culture, we first confirmed that the effect of an addition of potassium chloride to the culture had hardly any effect on the glycerol consumption and the CA production pattern (Fig. 4.3 a). The addition of CA to concentrations of 0.4 \(\text{g/L}\), which had been obtained in previous cultivations as final titers, visibly interfered with the CA degradation kinetics, though not with the glycerol consumption (Fig. 4.3 b). Remarkably, here the maximum CA titer is approximately the sum of the maximum CA titer of the culture without CA addition and the additional 0.4 \(\text{g/L}\). This could suggest, that a CA addition at this level does essentially not interfere with the strain’s physiology and productivity.

Adding more than 0.4 \(\text{g/L}\) of CA changed this picture (Fig. 4.3 c, d): Glycerol consumption decreased considerably, and at the highest concentration of CA added, almost no glycerol was consumed anymore by the cells, indicating that they were severely harmed. When identical amounts of CA were added after 18h, the observed effect was not that pronounced anymore, however a decreasing glycerol consumption rate could still be monitored with increasing CA concentrations.
Figure 4.3: Influence of different amounts of potassium clavulanate on the time course of CA and glycerol concentration in *S. clavuligerus* cultures. a) Control culture (open symbols) and culture supplemented with 20mM KCl at \( t = 0 \) (closed symbols). b-d) CA in the form of potassium clavulanate was added to final concentrations in the medium of 0.4, 1.5 and 4 \( \text{g/L} \) at \( t = 0 \)h (open symbols) and \( t = 18 \) h (closed symbols). In the latter case, the first sampling data point was not connected with the origin in order to not suggest a CA production by the bacterial cells.
The effect of CA on the CA production rate itself for the addition of 1.5 and 4 $\text{g/L}$ is more difficult to investigate than the effect on the glycerol consumption rate due to the simultaneous decomposition of CA and due to the fact that only a small increase due to production had to be determined against a large background of added CA. The decomposition is known to be a complex process, which is influenced amongst others by the microorganism itself and cannot therefore be accounted for by e.g. a simple mathematical model (Mayer and Deckwer, 1996; Roubos et al., 2002). However, the measured data showed that the addition of 0.4 $\text{g/L}$ at both time points did not decrease the production rate. For the addition of 1.5 $\text{g/L}$ or 4 $\text{g/L}$ at $t = 0$ no net production of CA could be observed in contrast to the experiments were CA was added at $t = 18 \text{h}$.

The OTR measurements gave a more detailed picture of the cultivations (Fig. 4.4) and broadly supported the above mentioned findings. The shaken control culture (Fig. 4.4 a) was again characterized by two distinct growth phases, and a sharp decrease in OTR after 40 h coincided with the exhaustion of glycerol. The addition of 0.4 $\text{g/L}$ CA at the beginning of the cultivation led to a similar growth pattern, however with smaller OTR$_{\text{max}}$ values in both phases and a delay of the complete depletion of glycerol. The addition of CA to 1.5 $\text{g/L}$ at the beginning of the cultivation gave a distinctly different growth profile: the OTR profile did no longer exhibit the bimodular pattern and increased much more slowly, which is in agreement with a much reduced glycerol consumption (Fig. 4.3 c). The culture with 4 $\text{g/L}$ of CA showed even lower OTR values that remained always below 0.1 $\text{g/Lh}$.

Similar, though not identical results could be obtained with the stirred cultures (Fig. 4.4 b). Most prominently, the culture with 1.5 $\text{g/L}$ of CA added at the beginning developed a considerable OTR towards the end of the cultivation, similar in size to the culture with only 0.4 $\text{g/L}$.

4.4 Discussion

Product inhibition of biological production systems is an important and widely observed phenomenon with prominent implications for the design and ultimately the success of biotechnological processes. Preliminary
Figure 4.4: Influence of CA addition on the OTR of *S. clavuligerus* cultures. CA was added to concentrations of 0.4 (low), 1.5 (medium) and 4 s/L (high), respectively. A) CA was added at t=0 h in shaken cultures. B) CA was added at t=0 h in stirred cultures (stirrer 2).

observations (Roubos et al. 2002) have for example already indicated that increased concentrations of the important β-lactamase inhibitor CA might lead to severe reductions in the specific growth rate of the producing *S. clavuligerus*. The overall inhibitory effect is frequently influenced by a multitude of cellular functions, as was recently shown for the mechanisms of ethanol tolerance in yeasts (Alper et al., 2006) and
as it has been broadly established e.g. for solvent tolerant and antibiotic resistant bacteria (Kumar and Schweizer, 2005; Ramos et al., 2002). Consequently, it appears most straightforward to exploit rather global indicators of cellular performance for measurement. One such indicator is cell dry weight, but this specific indicator is of little use in the context of antibiotics production, as many production phases in biotechnological processes start only after the specific cell growth rate has significantly decreased. Alternative global parameters that are easily measurable are carbon source and oxygen consumption. The former parameter will require either off-line measurements or an elaborate multiplexed on-line analytics, so it is probably less attractive for parallelized efforts. The determination of oxygen consumption, in contrast, is easy to parallelize and systems are available (Anderlei et al., 2004), including one based on a respirometric measurement principle that has been implemented in our laboratory (Chapter 3). It addresses cultivations in the scale of 50mL, which is a convenient volume for investigations with filamentous organisms, as it allows a considerable degree of parallelization and repetitive sampling, but avoids problems frequently associated with filamentous organisms in microtiter plates, such as excessive cell growth on the wall and the lack of comparability of growth rates and duration of lag phases. (Minas et al., 2000). Consequently, we wanted to validate oxygen consumption as a suitable indicator of product inhibition, specifically for the effect of increased clavulanic acid concentrations on S. clavuligerus, by comparing it to glycerol consumption, and thus open the route to highly parallelized investigations in this area.

4.4.1 Similarity of cultivation conditions over different cultivation scales and power input modes

As the overall behavior of bacterial cultures in general and their production behavior in particular is heavily influenced by the cultivation conditions applied (compare Fig. 4.1 a and 4.2 f), it is of prime importance to ensure their comparability during downscaling. Consequently, we compared first different cultivation schemes: aerated stirred tank fermenter and shaken and stirred small volume cultures. The cultivations in the aerated stirred tank fermenter compared well
with previous work which reported maximum CA concentrations of 0.2 - 0.3 g/L and biomass concentrations in the range of 8 - 12 g/L when employing similar media (Chen et al., 2002; Mayer and Deckwer, 1996). OTR data for laboratory scale fermentations of S. clavuligerus are seldom reported, but are estimated to be in the range of 0.3 - 1.3 g/Lh for different media (Belmar-Beiny and Thomas, 1991; Rosa et al., 2005; Roubos et al., 2001). Data on industrial S. clavuligerus fermentations suggest higher biomasses of up to 25 g/L (Neves et al., 2000) but moderate OTR\textsubscript{max} values of 0.8 g/Lh (Neves et al., 2001). In summary, the cultivations in the aerated stirred tank fermenter in this work are apparently well suited to serve as a reference to compare to smaller volume cultivations.

Overall, the growth, glycerol consumption, and production pattern obtained in the aerated stirred tank fermenter could be well reproduced on smaller scale for baffled shaken or stirred flasks, provided sufficient oxygen availability was ensured (Fig. 4.1, compare also (Rosa et al., 2005)). Where this condition was met, in particular the reproducibility of the glycerol consumption profile was high. The OTR profiles of the smaller scale cultures differed somewhat from the larger system and between themselves (Fig. 4.2), but showed the same major trends as the reference system. Such differences can possibly be explained in terms of the different power input levels and modes. These can influence cell morphology, which for filamentous organisms is known to play a role in productivity (Papagianni, 2004). Unfortunately, the influence of these factors specifically on S. clavuligerus has not been conclusively investigated, as previous works found higher lysis rates at increased gassed power inputs (Roubos et al., 2001), no influence of stirrer speed on productivity (Belmar-Beiny and Thomas, 1991), a productivity optimum when varying tip speed (Large et al., 1998), or the highest productivity at the highest tip speed (Rosa et al., 2005), so that we cannot safely establish this as a reason for the observed differences.

### 4.4.2 Influence of CA on S. clavuligerus cultures

After validating the comparability of cultivation modes, we investigated the effect of increased CA levels on S. clavuligerus behavior, specifically on glycerol consumption as a global parameter for the physiological state
of the culture. A dose dependent inhibitory effect of CA on glycerol consumption could be clearly observed, which was much more pronounced if CA was added at the beginning of the fermentation (Fig. 4.3, 4.4). This confirms that *S. clavuligerus* is indeed subject to product inhibition by CA, or given the ease with which CA is degraded in fermentation broths, one of the degradation products of CA. However, the concentrations at which a significant negative influence occurred in our experiments are much higher than can presently be reached in batch fermentations with the wild-type strain. While it is clear that in industrial fermentations much higher CA concentrations are achieved, it remains unclear whether these overproducing strains also exhibit a higher antibiotic resistance, and if so, to which extent. An inhibition of CA synthesis at the level of the current maximum wild-type titer of 0.4 g/L could not be observed, as CA still continued to accumulate in the medium after addition of CA.

### 4.4.3 Equivalence of glycerol consumption and OTR measurements as global parameters

Glycerol and oxygen consumption, the latter measured as OTR in the various cultures, lead consistently to identical interpretations of the physiological state of the *S. clavuligerus* cultures. The OTR can be continuously and rather accurately acquired and is therefore considerably more informative than glycerol consumption, for example in the description of different growth phases (compare the bimodal OTR profile in Fig. 4.2). Still, when compared to reference profiles, the adverse impact of high concentrations of CA on strain physiology can be clearly identified (Fig. 4.4). However, it might be left to the specific experiment to decide when a profile is indicative of reduced strain performance - in our experiments, the addition of 0.4 g/L of CA at the begin of a cultivation did not significantly change glycerol consumption and CA production, but did change the OTR profile. This would be in agreement with the interpretation that the accumulation of CA beyond the level of 0.4 g/L leads for example to slightly increased maintenance requirements for the strain, which in turn would be indicative of a beginning product inhibition. However, if applicable, the effects are too subtle to be noticed by the other two criteria.
In summary, we have shown that increased levels of CA severely interfere with the physiology of the producing strain at least at and above 1.5 g/L of CA. Consequently, either in situ product removal or addressing the multiple mechanisms of increased product resistance need to be considered when the efficiency of CA production processes is to be increased. At the same time, we could demonstrate that OTR measurements are an adequate parameter to evaluate the impact of CA on the producing cells, so that future experiments to this end can draw on available parallelized cultivation platforms.

**Acknowledgement**

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Chapter 5

Influence of clavulanic acid decomposition products on Streptomyces clavuligerus cells

Abstract

Production and decomposition of the β-lactamase inhibitor clavulanic acid (CA) occur simultaneously in Streptomyces clavuligerus cultures, limiting the maximal titers in fed-batch fermentations to about 0.5 g/L. We recently investigated if product-toxicity effects of CA itself are responsible for the low yields and could show that CA indeed had a negative influence on glycerol and oxygen consumption rate of S. clavuligerus cultures, especially at concentrations beyond 1.5 g/L. However, it remained unclear whether CA itself or its decomposition products, a com-
plex mixture containing amongst others several pyrazine derivatives, were responsible for the observed effects. To clarify this point, we supplemented *S. clavuligerus* cultures with i) mixtures of the decomposition product, ii) isolated and purified pyrazine derivatives stemming from this mixture or iii) with non-substituted pyrazine. In all cases, oxygen and glycerol consumption rates were altered, but CA production was not negatively influenced. Compared to the rather drastic effect of CA itself, it became clear, that the degradation product of CA exert a much less severe effect on growing *S. clavuligerus* cultures.

### 5.1 Introduction

Clavulanic acid (CA) is a potent inhibitor of β-lactamases produced by some strains of pathogenic microorganism resistant to β-lactam antibiotics thereby enabling efficient treatment of infectious diseases (Reading and Cole, 1977). It is produced by *Streptomyces clavuligerus* cells, yielding final titers between 0.1 and 1 s/l for wild-type strains. We recently investigated if these relatively low titers might be due to product inhibitory effects of CA by adding different amounts of CA to the growing cultures at different time points (chapter 4). It could be shown that the addition of CA had indeed a negative influence on the glycerol and oxygen consumption rates of the cultures. However, as CA has a poor chemical stability and decay takes place simultaneously with production (Mayer and Deckwer, 1996), it might as well be possible that - instead of CA - the decomposition products caused the reported effects. The hydrolysis of CA ultimately leads via the reactive intermediate 1-amino-2-oxo-butan-4-ol (2) to three different pyrazines: 2,5-bis(2-hydroxyethyl)pyrazine (3), 3-carboxyethyl-2,5-bis(2-hydroxyethyl)pyrazine (4) and 3-ethyl-2,5-bis(2-hydroxyethyl)pyrazine (5), (Fig. 2.1) (Finn et al., 1984; Haginaka et al., 1985). Pyrazine derivatives are potentially biologically active molecules, showing e.g. antimycobacterial (Gordin et al., 2000; Seitz et al., 2002), antimicrobial (Bonde and Gaikwad, 2004), modest antifungal (El-Emary et al., 1998), or cell growth inhibitory (Pettit et al., 1988) activity. We therefore also investigated the effect of various isolated CA decomposition products and mixtures of them on growing *S. clavuligerus* cells.
5.2 Materials and Methods

Preculture routines and medium composition were identical to previously described experiments (section 4.2.4). Unless mentioned otherwise, OTR measurements were performed in 25 mL cultures, stirred at 650 rpm with the custom-made magnetic stirrer (section 4.2.2), with the previously described Sapromat system (section 4.2.1). Samples for CA and glycerol determinations were withdrawn from separate 20 mL cultivations performed in shaken 100 mL Erlenmeyer flasks with 4 baffles, which generally gave similar cultivation results. Glycerol, CA and decomposition products were analyzed by standard HPLC protocols (section 4.2.6). All cultivations were performed in duplicates if not mentioned otherwise.

5.2.1 Influence of mixtures of CA decomposition products on *S. clavuligerus* cultures

A stock solution containing 48 g/L potassium clavulanate (American Laboratories, San Bruno, USA) in production medium was sterile filtered. The solution was incubated at 30 °C for 21 days. Complete decomposition of CA was confirmed by HPLC measurements. Different amounts of this stock solution were added to the cultures at the start of the cultivations and thereby diluted 1:100, 1:25 and 1:10 corresponding to equivalent CA concentrations of 0.4 (termed low), 1.6 (medium) or 4 g/L (high). Alternatively, an identical stock solution (see above) was incubated at 30 °C for 48 h to obtain a solution of CA and its degradation products. Different amounts of this stock solution were added to the cultures at the start of the cultivations (see above) corresponding to originally CA concentrations of 0.4 (low), 1.6 (medium) or 4 g/L (high).

5.2.2 Isolation and quantification of pyrazine derivatives

Compounds (3) and (5) were isolated from the decomposition product mixture by preparative HPLC by adapting the analytical protocol (see
section 2.2.7) as follows: the flow rate was set to \(10 \text{ mL/min}\) and all indicated times in the eluent protocol were scaled by a factor of 6 which represents the observed shift in retention times. The mixture was fractionated by employing a Prontosil 120-10-C18 AQ 10.0\(\mu\text{m}\) column (20 x 250 mm) (Bischoff Chromatography, Leonberg, Germany) and fractions containing pyrazines (3) and (5) were collected manually. Compounds were isolated from these fractions by removing the solvent under vacuum in a rotary evaporator and drying in a desiccator yielded brownish amorphous solids. Purity of the isolated substances was determined by HPLC to 90% (by peak area) employing the described analytical protocol (section 2.2.7). To enable pyrazine quantification by HPLC the exact concentration of the HPLC standard solutions containing the isolated pyrazines were determined by measuring the extinction with a photometer (Lambda 25, Perkin Elmer, Waltham, MA, USA) at 278 nm and calculating the concentration using literature extinction coefficients (Finn et al., 1984). For substance (4) no extinction is published, therefore the value published for pyrazine (5) was employed.

5.2.3 Influence of isolated pyrazines on \textit{S. clavuligerus} cultures

The small amount of isolated substance required us to limit the number of experiments that could be performed with these compounds. Specifically, the influence on the OTR profile of growing \textit{S. clavuligerus} cultures could only be investigated once in 100 mL flasks filled with 10 mL medium and stirred with 650 rpm with a 40 mm stirrer bar. The concentration of pyrazine (3) was 5.5 mmol/L (corresponding to 0.92 g/L), and of pyrazine (5) 4.5 mmol/L (0.88 g/L).

Furthermore we also investigated the effect of un-substituted pyrazine on \textit{S. clavuligerus} cultures. Pyrazine (Fluka, Buchs, Switzerland) was dissolved in water to a concentration of 20 mmol/L and sterile filtered. Sterile water, pyrazine stock and 2-fold concentrated production medium were combined so that the resulting medium contained pyrazine to one of three different levels, 1, 4 or 10 mmol/L. Culture conditions as described above (section 4.2.4) were applied.
5.3 Results

The decomposition of CA, occurring simultaneously with its production, is a complex process leading via the amino ketone (2) to different pyrazines (Fig. 2.1). The yields for pyrazines under neutral conditions depend on the starting concentration and a considerable amount of by-products is formed as well (Fig. 2.5, Tab. 2.2). The structures of the by-products have not yet been identified and it is therefore not possible to quantify them. Furthermore, the prevalent concentration of the reactive intermediate (2) cannot be determined spectrophotometrically. These two points make the accurate investigation of the influence of the various decomposition products difficult. However, we designed the experiments in such a way that we could distinguish at least between the effects of CA itself and its decomposition products.

5.3.1 Influence of completely degraded CA on S. clavuligerus cultures

First, we investigated the effect of completely degraded CA on S. clavuligerus cells. The decay reaction was performed in fermentation medium at 30 °C with a high starting concentration. The total pyrazine yield amounted to 50% and the stock solution contained 10.7 mmol/L (3), 11 mmol/L (4) and 31 mmol/L (5). Different amounts of this stock solution of mixed decomposition products were added at t = 0 to S. clavuligerus cultures corresponding to initial CA concentrations of 0.4, 1.6 and 4 μg/L, respectively, and CA production, glycerol consumption and OTR were monitored (Fig. 5.1). The OTRs measured for cultures supplemented with degradation products of CA showed the typical sharp decrease of OTR due to glycerol depletion at later time points than the one of the control culture (Fig. 5.1 a). The OTRₘₐₓ in the first growth phase decreased with increasing amounts of degradation products. Also in the second growth phase, the control culture reached the highest OTRₘₐₓ value, however the differences to the other cultures were rather small. The results obtained by the OTR measurements were confirmed by the glycerol quantification: The addition of increasing amounts of
decomposition products led to slightly decreased glycerol consumption rates but also to slightly increased maximum CA titers. This maximum was measured for all four cultures after 48 h, where at the same time the glycerol was completely metabolized (Fig. 5.1 b).
5.3.2 Influence of pyrazine (3) and (5) on *S. clavuligerus* cultures

Next, we tested the influence of the isolated pyrazines 3 and 5 on the OTR of *S. clavuligerus* Sapromat cultures (Fig. 5.2).

![Graph showing influence of pyrazines on OTR](image)

**Figure 5.2:** Influence of (3) and (5) on OTRs of *S. clavuligerus* cultures in 10 mL stirred flasks.

The limited availability of the compounds together with the aim to experiment with high concentrations allowed only a culture volume of 10 mL, which led to a different OTR profile already in the control culture. The maximum OTR of 0.5 g/Lh was reached already after 35 process hours and decreased slightly afterwards until glycerol depletion was indicated by the sharp drop in the OTR after 58 h. This behavior could be an indication for an oxygen limitation of the culture. In contrast to the control, both cultures with pyrazine 3 and 5, respectively, did not show a long transition phase between the two distinct growth phases in which the OTR was decreasing. Instead the OTR rose further until the maxima of 0.5 g/Lh (pyrazine (3)) and 0.45 g/Lh (pyrazine (5)) are reached. Glycerol depletion occurred in the culture supplemented with pyrazine (3)
after 53 h and after 60 h for the culture containing pyrazine (5). HPLC measurements confirmed that the pyrazines were not consumed during the cultivations (data not shown).

5.3.3 Influence of pyrazine

In order to obtain more information on the influence of pyrazines on *S. clavuligerus* cultures, we performed several cultivations supplemented with different concentration of the readily available non substituted pyrazine (Fig. 5.3). A distinct effect on the OTR of the cultures could be observed (Fig. 5.3 a): the higher the pyrazine concentration, the lower was the OTR\textsubscript{max} in both growth phase, but especially in the second one. Furthermore glycerol depletion occurred later if pyrazine was added to the medium. However, CA production was not markedly influenced by the pyrazine addition, only for the highest concentration a slightly lower maximum CA concentration was reached (Fig. 5.3 b). The CA production rate in the first 40 process hours was even higher for the cultures supplemented with pyrazine but then decreased earlier than in the control culture.

5.3.4 Influence of partly degraded CA

Finally, we examined the influence of partly degraded CA on *S. clavuligerus* cultures. The reasoning behind this experiment was that under these conditions the amount of reactive intermediates should be higher because the subsequent reactions to pyrazines were not supposed to have finished at this time point. Only 75% of the initial CA were degraded over the incubation period selected for the production of the solution with the partially degraded CA. HPLC analyses of the mixture at the start of the experiment showed that the pyrazine yield amounted to approximately 30%, based on the already degraded CA, which was lower than the expected final yield of around 50% (see above). In the experiment with the highest concentration of degradation products, the pyrazine content increased from $2.2 \text{ mmol/L}$ at $t = 0$ at the beginning of the cultivations to $3.3 \text{ mmol/L}$ after 63 h to $4.3 \text{ mmol/L}$ after 21 days.
The addition of partly degraded CA to *S. clavuligerus* cultures led to altered OTR profiles and lowered glycerol consumption rates (Fig. 5.4) with a decrease in OTR$_{\text{max}}$ that depended on the amount of partly degraded stock solution added. However, CA production was not inhibited, a net production of CA of 0.3 - 0.4 g/l was achieved in all cultivations.
Figure 5.4: Influence of partly decomposed CA. Different amounts of mixture of CA and decomposition products corresponding to originally present concentrations of CA of 0.4 (low), 1.6 (medium) or 4 s/L (high) were added to the cultures and OTR (a), CA (b, open symbols) and glycerol (b, closed symbols) were measured.

5.4 Discussion

The mixed decomposition products of CA and the different pyrazines affected the culture characteristics. The glycerol consumption rates
decreased slightly in a concentration dependent manner and the OTR profiles showed lower maxima, if cultures were supplemented with the various products. The OTR profiles of the cultures with isolated pyrazines are difficult to interpret, because under the prevailing conditions even the control culture showed an unusual OTR profile, which might be the result of an oxygen limitation. The isolated pyrazines altered especially the transition phase between the two growth phases, which essentially was no longer present. The glycerol depletion point was in the same range for all three cultures, showing that the pyrazines did not lead to obvious cell toxic effects.

However, the altered OTR profiles and glycerol consumption rates did not translate into a lowered CA production. It even seemed that the addition of pyrazines led to slightly earlier induction of CA production compared to the control cultures (Fig. 5.1, 5.3). This effect was especially pronounced for the cultures supplemented with the completely degraded CA. The experiments with the partly degraded CA furthermore showed, that CA concentrations of up to 1 s/l did not have feedback inhibitory effects on CA biosynthesis.

Comparing the results of this chapter to the rather drastic effects of CA itself (chapter 4), it becomes clear that the degradation products of CA - at least in the concentrations that arise in the course of CA decomposition - exert a much less severe effect on growing *S. clavuligerus* cultures and their CA production.

The observation that altered OTR profiles did not lead to an altered productivity indicates that OTR measurements alone are not sufficient for such complex cultivations of secondary metabolite producing organism. The measurements should be accompanied by sampling for product quantification. This implies that the current trend to cultivation scales smaller than 5 mL might not be helpful for process development efforts in the area of antibiotics, as the volumes might simply become too small for sampling. Parallel cultivation systems in the intermediate scale like the Sapromat or RAMOS (Anderlei et al., 2004) are therefore a necessary and reasonable supplementation of the equipment available on the microtiter scale.
Chapter 6

Ion exchange-based adsorption for *in situ* clavulanic acid removal in fed batch fermentations of *Streptomyces clavuligerus*

Abstract

The production of the β-lactamase inhibitor clavulanic acid (CA) is hampered by its accompanying degradation in *S. clavuligerus* fermentations. Thus, we investigated the potential of an *in situ* product removal (ISPR)
strategy to protect CA from degradation. CA could be immobilized effectively on the thermostable strongly basic ion exchange resin Diaion TSA1200. However contact times had to be kept short due the limited stability on the resin as well. In the developed ISPR protocol, cell free cultivation broth was treated twice a day for 5 min with resin, which allowed the net formation of a total CA concentration of 0.77 g/L compared to 0.35 g/L in the control culture. By feeding a soybean extract after each adsorption step, the net formation of CA could be further increased to 1.67 g/L, which was 3.2 times higher than in the corresponding control culture. Besides the prevention of CA decomposition in the fermentation broth, ISPR might have as well have stimulated the CA production rate by preventing subtle product inhibitory effects, as judged by the elevated glycerol consumption rate in the ISPR cultures.

6.1 Introduction

In situ product removal (ISPR) covers methods that lead to the selective removal of product from the site of its formation while the reactions continue. One effect of ISPR in bioprocesses is to prevent the product’s prolonged interaction with cellular and medium components, which otherwise might lead to increased product losses due to unfavorable environmental conditions such as temperature and medium compositions (Freeman et al., 1993). In the case of clavulanic acid (CA) production in cultivations with *S. clavuligerus*, the typically employed cultivation temperature, medium components that catalyze or lead to degradation, and the cells themselves accelerate the unwanted decomposition of CA compared to pure solution, leading to reduced yields (Mayer and Deckwer, 1996; Roubos et al., 2002). Consequently, we aimed at investigating ISPR strategies to remove the CA from the broth. Potential methods to remove charged molecules such as CA, which is characterized by a pKₐ of 2.5 (Plumb, 1991), are ion exchange adsorption, electrodialysis and reactive extraction (Stark and von Stockar, 2003). As it is technically most feasible and furthermore a standard process step during the downstream processing of CA (Reading and Cole, 1977), we specifically explored the potential of ion
6.2. MATERIALS AND METHODS

exchange adsorption as a potential means to remove CA. Generally, ion exchange adsorption as ISPR method is rarely reported, though often it is the method of choice to remove organic acids. Actually, in one of the first ISPR processes ever, salicylic acid was extracted in situ from P. aeruginosa growing on naphthalene by addition of the ion exchange resin Amberlite IR-400, thereby achieving a 5-fold increase in product yield (Kitai et al., 1968). Furthermore several successful examples of ion exchange utilization in in situ lactic acid removal were described (Gonzalez-Vara et al., 2000; Senthuran et al., 2004; Srivastava et al., 1992) as well as an improved hexanoic acid production process (Roddick and Britz, 1997), for which in situ ion exchange adsorption was shown to be biocompatible and to enable yield and productivity improvements. Previous examinations showed that CA can be adsorbed very effectively on the ion-exchange resin IRA-400. They also showed that CA is unstable on this resin (Mayer and Deckwer, 1996). This observed instability has of course to be reflected in the design of an ISPR treatment to make it successful. A standard ISPR concept would include incubation of the fermentation broth together with the resin or a continuous circulation of the cell free supernatant through a column, with the elution of the CA at the end of the fed-batch cultivation. However, taking into account a fermentation time of approximately 120 h, most of the CA would be degraded in the end. Consequently, one needs to consider a process where the contact time of CA and resin is kept very short. This could involve for example twice daily treatment of the cultivation supernatant for approximately 5 min with the resin and then continuation of the cultivation. Such a step-by-step strategy has also been applied by Lonza (Visp, Switzerland) in their process for the removal of 3-pyridylacetic acid (Glockler and Roduit, 1996).

6.2 Materials and Methods

6.2.1 Determination of adsorption isotherms

In order to determine adsorption isotherms of CA in pure water, 0.125 g of the strongly basic ion exchange resin Diaion TSA1200 (Resindion
S.R.I., Binasco, Italy) was washed twice with 10 mL of 1 M NaCl solution and three times with 10 mL H₂O. The pretreated resin was then gently shaken with 10 mL of CA solutions in the concentration range of 0.1-7 g/L at 28 °C for 30 min, which allowed equilibration of the system. For the determination of adsorption isotherms in production medium (see below), the amount of resin was tripled, as a lower adsorption capacity was expected. CA quantification in the supernatant allowed to determine the equilibrium concentration ([CA]_{eq}). The amount of CA adsorbed to the resin was calculated from a mass balance. The corresponding equilibrium adsorption capacities q_{eq} were expressed in g CA per g of adsorbent. Langmuir isotherms of the form

\[
q_{eq} = q_{max} \frac{K \cdot [CA]_{eq}}{1 + K \cdot [CA]_{eq}}
\]  

(6.1)

were fitted to the datasets with IGOR (Wavemetrics, Inc., Portland, USA). A single adsorption site and single adsorbing species Langmuirian model was also used when the adsorption of CA from medium rather than water was investigated. Consequently, the model was only used as a convenient mathematical description, not to suggest adsorption mechanisms.

6.2.2 Adsorption kinetic

To determine the time required to reach the adsorption equilibrium, 5 g of resin Dianion TSA1200 were incubated with 100 mL of a 0.9 g/L CA solution in water at 28 °C in a shaking hood. Aliquots of 1 mL were withdrawn regularly and analyzed for remaining CA.

6.2.3 Stability of CA immobilized on Dianion TSA1200

In order to determine the stability of CA immobilized on Dianion TSA1200, the adsorption was carried out in two ways: in the batch mode and in the dynamic mode. In the batch mode, CA was adsorbed
onto 0.25 g ion exchange resin by gently shaking the resin with 10 mL of a CA solution of 1 g/L for 30 min at 28 °C. The supernatant was withdrawn and the loaded resin was stored at 28 °C for the indicated time until the elution was performed. For this, the resin was incubated twice with 10 mL of a 1 M NaCl solution for 10 min. The two elution fractions were pooled and analyzed for their CA content.

For the dynamic mode a plug column (Omnilab AG, Mettmenstetten, Switzerland) of 150 mm length and 7.5 mm inner diameter was packed with pretreated Dianion TSA1200. The column was connected to a HPLC system (see below) and a 0.17 g/L CA solution was pumped through the column. The flow rates and adsorption times were set to 4 mL/min for 4 h, 2 mL/min for 8 h, and 1 mL/min for 16 h. The eluent was monitored continuously with a UV/Vis detector set to a wavelength of 210 nm to detect CA in the outflow. After that the column was flushed with 1 M NaCl solution at a flow rate of 2 mL/min until the UV-signal indicated that CA was no longer eluted from the system. The outgoing flow was collected and analyzed for CA. The fraction of recovered CA was calculated by a mass balance. It should be noted that CA decomposition in the feeding solution was shown to be negligible and that the adsorption on the column was quantitative.

6.2.4 S. clavuligerus cultivation with repeated product removal by ion exchange adsorption

Preculture routines for S. clavuligerus DSM 738 (DSMZ, Braunschweig, Germany) were performed as previously described (section 4.2.4). For the main cultures the production medium contained no buffer salts because they would interfere with the ion exchange adsorption. Instead the pH was measured manually with pH paper at every sample point and maintained between 6 and 7 by addition of 1 M NaOH or 1 M H₂SO₄. In all cultures glycerol was fed discontinuously in aliquots at the sampling points, as indicated by sharply rising glycerol concentrations in the corresponding figures. The 50 mL cultures were aerated by stirring at 650 rpm with stirrer 2 (section 4.2.2). The adsorption steps in the ISPR cultures were carried out strictly under sterile conditions as follows: The complete culture was transferred into a 50 mL falcon...
tube and centrifuged for 3 min at 4°C with 3200 g (Centrifuge 5810 R, Eppendorf, Hamburg, Germany). The supernatant was then transferred to another 50 mL falcon tube containing 5 g of heat sterilized Diaion TSA1200 and incubated for 3-5 min with gentle manual shaking of the tube. After that, the CA depleted medium and the cells were mixed thoroughly to brake up the pellet and the mixture was transferred back to the cultivation flask. The contamination of the culture with ion exchange resin was avoided by pouring the liquid through sterile 100 μm nylon sieves (BD Biosciences, Bedford, MA, USA). After that the resin was washed with 1 M NaCl, water, 70% ethanol and sterile water and reused for the next adsorption cycle. Alternatively, the experiment was carried out with additional feeding after each adsorption step: 1 mL of 4x soybean extract was added after each adsorption cycle. The 4x soybean extract was obtained by a volume reduction of the 1x soybean extract (section 4.2.4) by boiling. Control cultures were treated identically, but without the centrifugation and adsorption steps.

6.2.5 Analytical methods

CA and glycerol were quantified as described previously (section 4.2.6).

6.3 Results

We aimed at investigating the possibility of preventing loss of CA during production by applying an ISPR strategy based on ion exchange adsorption. As CA is characterised by a pK_a of 2.5 (Plumb, 1991), it is negatively charged at neutral pH and is able to bind to basic ion exchange resins.

First, we determined the adsorption isotherms for CA dissolved in water and production medium, respectively on Diaion TSA1200. This resin was chosen due to its thermal stability, which allowed standard heat sterilisation of the resin. Satisfactory fits were obtained based on the
Langmuir adsorption model, which allowed, though only in an empirical fashion, a convenient description of the adsorption (Fig. 6.1). The medium compounds reduced $q_{\text{max}}$ from $(0.19 \pm 0.01) \text{ s/g}$ for adsorption from water to $(0.053 \pm 0.003) \text{ s/g}$ for adsorption from medium. However, the adsorption constants $K_{\text{H}_2\text{O}}$ and $K_{\text{Medium}}$ were similar with values of $(2.05 \pm 0.36) \text{ L/g}$ for water and $(1.82 \pm 0.35) \text{ L/g}$ for medium. With these parameters it is now possible to estimate the required amount of adsorbent for the desired removal efficiency. This is visualized in Fig. 6.2, showing the fraction of immobilized CA as a function of the initial CA concentration in medium for a resin load of 1-10% (wt/vol). According to this, a resin load of 10% (100 s/L) is sufficient to immobilize 90% of the produced CA over a wide concentration range. A kinetic experiment showed that it took around 30 min to reach the adsorption equilibrium (Fig. 6.3). As the degradation of CA is an important element in any CA product removal scheme, we investigated whether the compound would
be stable when adsorbed to the chosen ion exchange resin. To this end, we designed two experiments; one in batch mode and one in the dynamic mode. In the batch test, CA was adsorbed, the supernatant was removed and the subsequent elution step was started after the indicated time. The retrieval rate decreased considerably, from 98% directly after adsorption to only 54% after 20 h (Tab. 6.1). Alternatively, we used a dynamic system, in which a CA solution was pumped through an ion exchange column and that was more similar to a residence time profile that one would obtain in a CA production by fermentation. Again, a decrease in retrieval was observed: in 16 h around 15% of the compound was lost. This clearly indicates that short contact times between CA and resin are necessary, preferably not longer than 1 h, as retrieval rates during the adsorption step should exceed at least 95%.

In order to investigate the effect of product removal on the productivity of the process, we investigated step-wise adsorption of CA to Diaion
Figure 6.3: Adsorption kinetic of CA in water on Diaion TSA 1200. For this experiment, 5 g of resin were contacted with 100 mL of a 0.9 g/L CA solution at 28 °C.

Table 6.1: Stability of CA immobilized on Diaion TSA1200

<table>
<thead>
<tr>
<th>Batch system</th>
<th>Dynamic system</th>
</tr>
</thead>
<tbody>
<tr>
<td>time adsorbed prior to elution</td>
<td>CA recovery</td>
</tr>
<tr>
<td>/h</td>
<td>/%</td>
</tr>
<tr>
<td>0</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>86</td>
</tr>
<tr>
<td>20</td>
<td>54</td>
</tr>
</tbody>
</table>

TSA1200 in batch culture (Fig. 6.4 a, b). The addition of 10% wt/vol of Diaion TSA1200 to the supernatant allowed to repeatedly reduce the
CA level in the supernatant, followed by increases in the CA level in the phases between the additions. The adsorbed fraction of CA was lower than 90%, which can be attributed to the short adsorption time, which does not allow to reach the adsorption equilibrium. In the control culture, the CA level remained approximately constant at around 0.35 s/L after 35 h. The interpretation of these results is complicated by the fact that CA is known to degrade in fermentation media, so it remains unclear to what extent the approximately constant level of CA in the control culture after 35 h is the result of an interplay of synthesis and degradation. Assuming for the time being that all the produced CA in the experiment could be recovered intact, then the repeated addition of IEX to the supernatant would have allowed to increase the amount of recoverable CA from one cultivation considerably, although at the expense of a prolonged cultivation period. In line with this theory, the confirmed total amount of produced CA of 0.77 s/L has been included in Fig. 6.4, which would correspond to a improvement by a factor of 2. The glycerol consumption was similar to the control culture.

The addition of resin to the culture medium was likely to also remove a series of medium components in addition to the CA. In fact, the repeated adsorption steps had a clearly visible effect on the culture: it was almost completely decoloured in the end and the final packed mycelial volume was decreased (Fig. 6.5). We therefore carried out a second product removal experiment in which this assumed loss was to be compensated by adding 1 mL of concentrated soybean extract after each adsorption step. This additional measure led to a further considerable increase in the confirmed amount of produced CA, which amounted to a total of 1.67 s/L (Fig. 6.4 c,d). This level was 3.2 times the level of the corresponding control experiment which had received the additions of soy bean extract but had not been treated with resin. Also the total amount of consumed glycerol was increased in the ISPR culture. The loss of biomass could be compensated; the final packed mycelial volume was comparable to the control culture without extract feed (Fig. 6.5).
6.3. RESULTS

Figure 6.4: Comparative fermentation of *S. clavuligerus* with and without *in situ* ion exchange adsorption. a) CA concentration and additive CA concentration and b) glycerol concentration and consumed glycerol in cultivations without extract feed. c) CA concentration and additive CA concentration and d) glycerol concentration in flasks and consumed glycerol in cultivations with extract feed. The symbols are for c as in a and for d as in b.
Figure 6.5: Packed mycelial volume at the end of the cultivations. Shown are from left to right the control culture without extract feed, the ISPR culture without extract feed and the ISPR culture with extract feed.

6.4 Discussion

We aimed at investigating the possibility to prevent the loss of CA during production by applying an ISPR strategy based on short contacts between ion exchange resin and CA. The short contact times were necessary, as the stability of CA immobilized on Diaion TSA1200 is low. However, limiting it to times below approximately 1 h allowed us to recover well beyond 95% of CA (Tab. 6.1).

A second problem in ISPR strategies is the occupation of the ion-exchange sites by medium components rather than the product, so that the capacity to remove CA from the fermentation broth might be reduced. This was in fact the case when CA adsorption was investigated together with fresh fermentation medium (Fig. 6.1). However, the decrease in adsorbed amount was tolerable, as still 90% of CA could be
6.4. DISCUSSION

adsorbed by applying a resin load of 10% wt/vol (Fig. 6.2). The adsorption of medium components also leads to a third problem of this strategy - the possible removal of essential or at least important (in terms of productivity) components from the medium. Again, indications for this problem could be observed in the small scale cultivations carried out here, when the cells resulting from the cultivation without extract feed but with resin addition resulted to be of a different consistency than without resin addition (Fig. 6.5). Furthermore, the amounts of CA produced per constant time decreased towards the end of the cultivation, indicating either an accumulating metabolite, an inhibiting intermediate, or in fact the removal of an important medium component. However, the repeated addition of a complex medium component could overcome the last mentioned problem (Fig. 6.4 c, d), indicating that this addition either prevented the metabolite from forming, made the cells more resistant against a potential accumulating intermediate, or simply compensated for a medium component removed with the resin. Whichever explanation (or whichever combination of explanations) will turn out to be the correct one, it is clear that the repeated removal of CA from the broth helps creating an environment in which CA production is considerably faster than its degradation. This might be a result of the reduced CA concentrations in the experiments with removal alone (CA degradation after all being a function of the CA concentration itself, see chapter 2), or it might be possible that the removal of an inhibitory effect exerted by the CA also stimulated its production rate, as might be concluded from the enhanced glycerol consumption rate of the extract supported culture with resin addition.

The presented results represent a clear motivation to pursue ISPR as a technology for yield increase in the future, after the different aspects raised in this work have been addressed. Especially, suitable protocols should be developed, how to handle the CA after the adsorption. The workup could be done either separately for each adsorption step, which is very time consuming, or alternatively it might be possible to cool or even freeze the immobilized CA prior to the pooled further processing. In the end, the isolated yields should be compared and a cost analysis should be performed to judge whether this process has a future on industrial scale.
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Chapter 7

Summary and conclusion

In this thesis the potential of an *in situ* product removal (ISPR) strategy to improve the production of the β-lactamase inhibitor clavulanic acid (CA) from *S. clavuligerus* cells was examined. Such an approach appeared reasonable i) because the yields of CA are limited due its accompanying decomposition during the production process and ii) because literature data suggested that CA exhibits cell toxic effects on its producer strain. Both problems would benefit greatly from a successful ISPR strategy. The following issues were addressed in this thesis:

The second chapter describes the results of kinetic investigations on the CA decomposition, which were performed for CA concentrations between 2.5 and 20 g/L, a characteristic range for an industrial CA production process. It was found that CA and its decomposition products catalyzed the CA hydrolysis and that the corresponding pseudo first order kinetic constants increased approximately three-fold within the investigated concentration range. A kinetic model was proposed, which was able to satisfactorily describe the CA decomposition process in buffered solutions.
In chapter 3 the adaptation of a 12-fold parallel medium scale fermentation device is discussed that allows the on-line measurement of oxygen transfer rates (OTR) of magnetically stirred cultures based on a respirometric principle. After optimization of the operating parameters, the device could accurately record common process development-relevant effects such as acetate formation, diauxic growth, and nutrient limitations in oxygen unlimited growing 50mL E. coli cultivations. The measuring principle was validated by comparing the obtained results with independently obtained cultivation data such as dissolved oxygen (DO), glucose or optical density and with simulated data based on parameters from literature.

For the desired investigations on the inhibitory effect of CA on S. clavuligerus cells, the oxygen transfer capacity of the device was improved further by employing custom made stirrers, as depicted in chapter 4. Now, results comparable to a laboratory scale aerated stirred tank reactor could be achieved. With this properly scaled-down device, which allowed to significantly reduce the volume under study while replicating rather well the conditions in a larger reactor aerated by air sparging, the effect of CA additions on the cultures was investigated. It was shown that increased levels of CA severely interfere with the physiology of the producing strain at least at and above CA concentration levels of 1.5g/L, as indicated by a dose-dependent decrease in maximal OTRs and glycerol consumption rates. However, inhibition of CA synthesis at the level of the current maximum wild-type titer of 0.4g/L could not be observed, as CA still continued to accumulate in the medium after addition of CA in this concentration range. In chapter 5, it was examined whether CA itself or its decomposition products, a complex mixture containing amongst others different potentially antimicrobial pyrazine derivatives, were responsible for the observed effects. To clarify this point, S. clavuligerus cultures were supplemented with i) mixtures of the decomposition product, ii) isolated and purified pyrazine derivatives stemming from this mixture, or iii) with non-substituted pyrazine. In all cases, oxygen and glycerol consumption rates were altered, but CA production was not negatively influenced. Compared to the rather drastic effect of CA itself, it became clear, that the degradation product of CA exert a much less severe effect on growing S. clavuligerus cultures.
Chapter 6 finally describes the development and application of ion exchange adsorption-based method for the in situ removal of CA from *S. clavuligerus* cultivations. CA could be immobilized effectively on a strongly basic ion exchange resin, but CA stability was limited in the adsorbed state. Thus an ISPR protocol was developed in which CA was removed batch-wise from the fermentation broth by treating it twice a day for a limited time with the ion exchange resin and keeping thereby the contact time short. This protocol led to a net production of 0.77 g/L of CA compared to 0.35 g/L in the control culture without ISPR. To compensate for nutrient removal, a soybean extract was intermittently fed and allowed the net formation of CA to further increase to 1.67 g/L, which was 3.2 times higher than in the corresponding control culture.

The presented results represent a clear motivation to pursue ISPR as a technology for yield increase in the CA production process in the future. Such an ISPR process needs to demonstrate its suitability on reactor scale, so the adsorption step needs to be integrated into the fermentation process. Only few examples exist where such integrations have been successfully implemented on technical scale, in particular for long fermentation processes as it is here the case (typical fermentation time 5 days). Furthermore, the beneficial effect of the adsorption strategy and in particular the effect of the removal of medium components and the compensation by extract feeding have to be confirmed on technical scale. Here, it would be helpful to more precisely understand which of the critical components is removed by the adsorption and design a more rational feeding protocol. Should these confirmations of the results be successful, there is ample potential to optimize the proposed ion exchange-based ISPR process for the production of CA with respect to duration, number and mode of adsorption cycles. Especially, suitable protocols should be developed how to handle the CA after the adsorption to avoid the subsequent decay outside the fermentation broth. The work-up could be done either separately for each adsorption step, which is laborious but allows to use reduced amounts of resin, or alternatively it might be possible to store the resins cool or even frozen prior to the pooled further processing. Our results indicate that successful implementation of an ISPR protocol would allow to significantly advance CA production in *S. clavuligerus* fermentations even with the wild-type strain.


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