Monitoring, Characterizing, and Influencing the Poly(γ-glutamic acid) Production by *Bacillus licheniformis*

Monitoring, Charakterisierung und Beeinflussung der γ-Polyglutaminsäureproduktion von *Bacillus licheniformis*

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Abstract

Bacillus spp. are used for the production of industrial enzymes, such as proteases for the detergent industry, but are also known to be capable of producing biopolymers such as poly(γ-glutamic acid) (γ-PGA). Biopolymers increase the viscosity of the fermentation broth, thereby impairing mixing, gas/liquid mass transfer, and heat transfer in any bioreactor system. Undesired biopolymer formation has a significant impact on fermentation and downstream processing performance, respectively. The first part of this thesis shows how undesirable γ-PGA formation of an industrial protease producing *Bacillus licheniformis* strain (A1) was prevented by switching the nitrogen source from ammonium to nitrate. The maximum viscosity at a shear rate of 300 s^{-1} was reduced by over 90 %, while enzyme production was not affected. Protease activities of 38 and 46 U mL^{-1} were obtained for ammonium and nitrate, respectively. The applicability was shown for shake flask and lab scale stirred tank bioreactor cultivations. With the presented results, protease production with industrial *Bacillus* strains is now possible without undesired γ-PGA formation and its negative impact on shake flask and stirred tank bioreactor fermentations.

On the other hand, *Bacillus* species are also used for the commercial production of the biopolymer γ-PGA. The second part of the thesis presents the characterization of the typical γ-PGA producing *Bacillus licheniformis* strain ATCC 9945 in shake flask cultivations under standard cultivation conditions from the literature. The cultivations were strongly oxygen limited, and after a certain time, the oxygen transfer rates of the parallel running cultures diverged. The respiration activity of some cultures ceased while others continued for several days. An early termination of respiration activity could always be attributed to a low pH value. Sample viscosity, which is an indirect measure of γ-PGA formation, was maximal after 24 to 48 h, thus preceding the parallel cultures' diverging. The observed non-reproducible growth, therefore, did not interfere with γ-PGA production. After its maximum was reached, the effective viscosity decreased severely, indicating the start of the enzymatic degradation of γ-PGA. The maximum of effective viscosity coincided with a depletion of citric acid and phosphate.

Different medium compositions and filling volumes were investigated for their influence on γ-PGA production. A higher initial citric acid concentration increased the maximum effective viscosity significantly while a higher initial phosphate concentration had no impact under standard conditions. More citric acid in combination with more phosphate was beneficial for the γ-PGA production. However, the increase of citric acid and ammonium concentrations at the same time led to low pH values and early termination of the respiration activity. By using different filling volumes, different levels of oxygen transfer capacities were induced, resulting in oxygen unlimited or limited conditions. The different oxygen transfer capacities were shown to exhibit a strong influence on growth and γ-PGA formation. It could be demonstrated that a certain level of oxygen limitation is useful for the γ-PGA production process.

In the third part of this thesis, online measurement of the volumetric power input is applied to follow γ-PGA formation and degradation continuously during a running cultivation. The online measured power input corresponded well to the offline measured effective viscosity. The determination of molecular weight and concentration of γ-PGA showed that viscosity and power input depended more strongly on changes of the molecular weight than of the concentration. The increase in viscosity due to a higher citric acid concentration or the supplementation of trace elements was also detectable with the online measured power input signal. Moreover, the power input measurement was useful to reveal different production patterns of γ-PGA producing strains, as was demonstrated by a comparison of the strains ATCC 9945 and A1. Finally, a correlation between offline determined viscosity and online measured power input was established which enabled the calculation of a continuous viscosity signal from the online measured power input.

Kurzfassung

Bei der Produktion industrieller Enzyme werden sehr häufig Arten der Gattung *Bacillus* eingesetzt. Diese *Bacillus*-Arten sind allerdings auch dafür bekannt, das Biopolymer γ-Polyglutaminsäure (γ-PGA) herstellen zu können. Die Bildung von Biopolymeren erhöht die Viskosität des Fermentationsmediums und beeinflusst die Durchmischung, den Wärmeaustausch und den Massentransfer zwischen Gas- und Flüssigphase im Bioreaktor. Eine unerwünschte Biopolymerbildung hat sowohl großen Einfluss auf die Fermentation als auch auf die anschließende Produktaufarbeitung. In dieser Arbeit wird gezeigt, wie die unerwünschte γ-PGA-Bildung eines industriellen *Bacillus licheniformis* Proteaseproduktionsstamms (A1) unterdrückt werden kann, indem als Stickstoffquelle Nitrat anstelle von Ammonium eingesetzt wird. Dadurch konnte die Viskosität des Mediums um über 90 % gesenkt werden, während sich die Proteaseaktivität durch den Austausch der Stickstoffquelle nicht veränderte. Es wurden Proteaseaktivitäten von 38 und 46 U mL^{-1} für Ammonium und Nitrat erreicht. Es wurde die Anwendbarkeit für Kultivierungen im Schüttelkolben und im 3 L Rührkessel gezeigt. Die erzielten Erkenntnisse ermöglichen es, in Zukunft industrielle Produktionsprozesse zur Proteaseherstellung mit *Bacillus*-Stämmen durchzuführen, ohne das Schüttelkolben- und Rührkesselfermentation durch unerwünschte γ-PGA-Bildung gestört werden.

Bacillus-Arten werden aber nicht nur zur Enzymproduktion, sondern auch zur industriellen Produktion des Biopolymers γ-PGA eingesetzt. Der zweite Teil dieser Arbeit beschäftigt sich daher mit der Charakterisierung eines typischen γ-PGA-produzierenden *Bacillus licheniformis* Stammes (ATCC 9945), der in Schüttelkolbenexperimenten unter sogenannten Standardbedingungen kultiviert wurde, die aus der entsprechenden Literatur übernommen wurden. Es zeigte sich, dass unter diesen Bedingungen eine starke Sauerstofflimitierung vorlag, und dass nach einer gewissen Kultivierungszeit die Sauerstofftransferraten (OTR) der bis dahin sehr ähnlich gelaufenen Kulturen unterschiedlich verliefen. Einige der Kulturen stellten ihre Atmungsaktivität vergleichsweise früh ein, während andere Kulturen noch mehrere Tage Atmungsaktivität zeigten. Ein frühes Einbrechen der OTR konnte dabei immer mit einem sehr niedrigen pH-Wert assoziiert werden. Die offline gemessene, effektive Viskosität diente als Maß für die γ-PGA-Bildung. Die maximale effektive Viskosität wurde nach 24 bis 48 h

gemessen. Das Aufspalten der OTR-Kurven der bis dahin gleich gewachsenen Kulturen trat erst anschließend auf. Das nicht reproduzierbare Verhalten der Kulturen hatte somit keinen Einfluss auf die Produktbildung. Nach Erreichen der maximalen effektiven Viskosität war immer ein deutlicher Abfall der Viskosität zu beobachten, der auf den Beginn des enzymatischen γ-PGA-Abbaus hinwies. Der Zeitpunkt der maximalen effektiven Viskosität korrelierte mit einem vollständigen Verbrauch von Zitronensäure und Phosphat.

Verschiedene Medienzusammensetzungen und Füllvolumina wurden im Hinblick auf ihren Einfluss auf die γ-PGA-Bildung untersucht. Eine höhere Zitronensäurekonzentration im Medium führte zu einer deutlichen Erhöhung der maximalen Viskosität, während eine höhere Phosphatkonzentration unter Standardbedingungen keinen Einfluss zeigte. Eine Kombination aus erhöhter Zitronensäure- und Phosphatkonzentration hatte hingegen einen zusätzlichen positiven Effekt. Die Kombination aus erhöhter Zitronensäure- und Ammoniumkonzentration führte jedoch zu niedrigen pH-Werten und daraus resultierend zu einem frühen Einbruch der OTR. Durch verschiedene Füllvolumina konnten unterschiedliche Niveaus einer Sauerstoff-limitierung bzw. nicht sauerstofflimitierte Bedingungen eingestellt werden. Die verschiedenen Bedingungen hatten einen starken Einfluss auf Wachstum und γ-PGA-Bildung, wobei ein gewisses Maß an Sauerstofflimitierung von Vorteil für die γ-PGA-Bildung war.

Im dritten Teil dieser Arbeit wurde ein Orbitalschüttler mit einem Drehmomentsensor zur Leistungseintragsmessung eingesetzt, um die γ-PGA-Bildung und den Abbau online zu verfolgen. Der online gemessene, volumetrische Leistungseintrag korrelierte hier sehr gut mit der offline bestimmten, effektiven Viskosität. Messungen des Molekulargewichts und der γ-PGA-Konzentration zeigten dabei, dass Viskosität und Leistungseintrag stärker von Änderungen des Molekulargewichts als von Konzentrationsänderungen beeinflusst wurden. Eine höhere Viskosität aufgrund einer höheren Zitronensäurekonzentration oder der Zugabe von Spurenelementen ließ sich mit Hilfe des Leistungseintragssignals nachweisen. Darüber hinaus zeigte der Vergleich der Stämme ATCC 9945 und A1, dass verschiedene Stämme unterschiedliche γ-PGA-Bildungskinetiken aufweisen können, die durch die Leistungseintragsmessung sehr gut erfasst wurden. Abschließend konnte eine Korrelationsgleichung aus den Daten der offline bestimmten Viskosität und des online gemessenen Leistungseintrags aufgestellt werden, die die Berechnung eines kontinuierlichen Viskositätsverlaufs aus den Leistungseintragsdaten ermöglicht.

Contents

Nomenclature

Abbreviations

γ-PGA	poly(γ-glutamic acid)
B. licheniformis	*Bacillus licheniformis*
B. subtilis	*Bacillus subtilis*
CDW	cell dry weight [g L^{-1}]
DOT	dissolved oxygen tension [%]
GPC	gel permeation chromatography
HPLC	high performance liquid chromatography
M_w	mass average molecular weight [kDa]
OD	optical density at 600 nm [-]
OTR	oxygen transfer rate [mmol L^{-1} h^{-1}]
RAMOS	Respiration Activity Monitoring System

Units

°C	degree Celsius
µL	microliter
µm	micrometer
cm	centimeter
g	gram
h	hour
kDa	kilodalton
L	liter
M	molar [mol L^{-1}]

mg	milligram
min	minute
mL	milliliter
mm	millimeter
mM	millimolar [mmol L^{-1}]
mol	SI unit of amount of substance
nm	nanometer
rpm	revolutions per minute
v/v	volume per volume
vvm	volume per volume and minute (aeration rate)
w/v	weight per volume

List of Figures

List of Tables

Chapter 1

Introduction

1.1 *Bacillus licheniformis* as production host

Bacillus licheniformis (*B. licheniformis*) is a gram-positive, rod-shaped, and peritrichously flagelatted bacterium of the family *Bacillaceae*. It is found ubiquitously in the environment and can be isolated easily from soil and the rhizosphere of plants. *B. licheniformis* forms heat-resistant endospores as a protection against extreme environmental conditions (Bisset and Street, 1973; Priest, 1989; Priest, 1993). *B. licheniformis* belongs to the so-called *B. subtilis* group (group II) of *Bacillus* species (Priest, 1993).

The natural habitat of *Bacillus* spp. contains a wide variety of carbohydrates derived from plants, animals, and microorganisms. Therefore, *Bacillus* species often secrete a large number of polysaccharide-degrading enzymes like α-amylase, pullulanase, pectate lyase, glucanase, and xylanase to break down polysaccharides into units which are small enough for transport into the cell (Deutscher et al., 2002; Veith et al., 2004). *B. licheniformis* is able to grow by anaerobic respiration using nitrate as an electron acceptor (nitrate respiration) and also by fermentation of glucose in the absence of exogenous electron acceptors. Fermentation products are acetate, 2,3-butanediol, lactate, formate, ethanol, succinate, and pyruvate, indicating a mixed-acid fermentation (Priest, 1989; Shariati et al., 1995).

B. subtilis is one of the best studied microorganisms, among the prokaryotes second only to *Escherichia coli*. It has been used as a model organism to investigate for instance physiology,

gene regulation, and metabolism of gram-positive bacteria, and also the mechanism of sporulation (Sonenshein et al., 2002). Since *B. subtilis* and *B. licheniformis* are closely related, many findings for the former are also true for the latter (Veith et al., 2004).

The ability of certain *Bacillus* strains to produce and secrete large amounts (20 to 25 g L^{-1}) of extracellular enzymes and proteins in general has put them among the most important industrial producers of homologous and heterologous proteins (Schallmey et al., 2004). They are also well known producers of antibiotics, nucleotides, bio-surfactants, biofuels, vitamins and biopolymers, such as poly(γ-glutamic acid) (Harwood, 1992; Kumar et al., 2013; Schallmey et al., 2004). One major product, which is manufactured in bulk quantities, is proteases for the detergent industry (Maurer, 2004). This industry accounts for more than one third of the industrial enzyme market and almost all detergent proteases are produced by *Bacillus* species, such as *B. licheniformis* and *B. subtilis*. In 2012, the market for industrial enzymes was estimated to be 3.9 billion US$ (Bedingfield, 2012).

1.2 Poly(γ-glutamic acid)

1.2.1 Properties and applications

Poly(γ-glutamic acid) (γ-PGA) is an anionic, water-soluble, biodegradable, edible, and nontoxic biopolymer. It consists of up to 10,000 D- and/or L-glutamic acid monomers and can be characterized by its molecular weight distribution and the ratio (in case of mixed γ-PGA) of D- to L-glutamic acid subunits. In contrast to proteins, where amino acids are bound by α-amide linkages, glutamic acid monomers in γ-PGA are linked by γ-peptide bonds (Fig. 1-1). This property makes γ-PGA stable against proteases. γ-PGA is mainly produced by *Bacillus* spp. and can either be degraded chemically, physically, or enzymatically by specific depolymerases. These depolymerases are often expressed by the producing organisms themselves. Microbially produced γ-PGA mostly has a high molecular weight in the range of 10-1,000 kDa and a high polydispersity (Bajaj and Singhal, 2011; Buescher and Margaritis, 2007; Shih and Van, 2001).

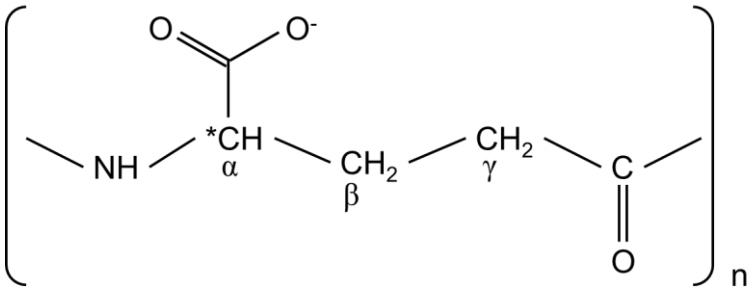

Figure 1-1 Chemical structure of poly(γ-glutamic acid)

Bacillus spp. are used for the commercial production of γ-PGA, but they may also secrete this biopolymer as an undesired by-product in other production processes. Originally known from the Japanese food natto, a great variety of possible applications for γ-PGA exists today: in food and skin care industries (Ho et al., 2006; Konno et al., 1989; Tanimoto, 2010), in wastewater treatment (Mark et al., 2006; Yokoi et al., 1996), in biodegradable plastics (Kubota et al., 1995), as cryo-protectant (Shih et al., 2003), and in the pharmaceutical industry as drug delivery systems, hydrogels, and surgical glue (Akagi et al., 2005; Buescher and Margaritis, 2007; Ho et al., 2005; Park et al., 2001).

1.2.2 Natural formation and synthesis pathway of γ-PGA

γ-PGA was first identified as a substance in the capsule around *Bacillus anthracis* cells, the pathogenic agent of anthrax (Ivánovics and Bruckner, 1937). Up to now, *B. anthracis* seems to be the only organism which produces pure D-γ-PGA. The capsule of γ-PGA protects the cells from the human immune system and can therefore be regarded as a virulence factor (Green et al., 1985). Later, other γ-PGA producing *Bacilli* were identified, which produced D/L-γ-PGA in a secreted form (Leonard et al., 1958; Thorne et al., 1954; Thorne and Leonard, 1958). Besides *Bacillus* species, a number of other species from all three domains - archaea, bacteria, and eukaryotes - have been shown to produce γ-PGA (Hezayen et al., 2001; Kocianova et al., 2005; Niemetz et al., 1997; Weber, 1990). Examples are listed in Table 1-1. In its secreted form, γ-PGA is assumed to serve as an extracellular nutrient storage, to enhance biofilm formation, and to act as a protection against the environment, e.g. against heavy metal ions, high salt concentrations, phagocytic attacks by predators, or phage infection (Itoh and Kimura, 2003; Kimura and Itoh, 2003; Kimura et al., 2004; Kocianova et al., 2005; Stanley and Lazazzera, 2005).

Table 1-1 Molecular size and stereochemical composition of γ-PGA produced by various organisms
Adapted from Ashiuchi and Misono (2002).

Producer	Molecular mass [kDa]	Content [%]	
		D-Enantiomer	L-Enantiomer
Bacillus subtilis natto	10-1000	50-80	20-50
Bacillus subtilis chungkookjang	> 1000	60-70	30-40
Bacillus licheniformis	10-1000	10-100	0-90
Bacillus anthracis	Not determined	100	0
Bacillus megaterium	> 200	50	50
Bacillus halodurans	10-15	0	100
Natrialba aegyptica	> 1000	0	100
Hydra	3-25	0	100

Among the γ-PGA producing *Bacillus* species are *B. subtilis* and *B. licheniformis*, of which several products have the GRAS (Generally Recognized As Safe) status of the FDA (U. S. Food and Drug Administration, 2015). Moreover, their genomes are completely sequenced (Kunst et al., 1997; Rachinger et al., 2013; Veith et al., 2004). Thus, they are ideally suited for academic research on and industrial production of γ-PGA.

The gene cluster encoding the γ-PGA synthesizing complex was first identified in *B. anthracis*, where it is located on a plasmid (Green et al., 1985; Uchida et al., 1985). In contrast, the homologue gene operons in *B. subtilis* and *B. licheniformis* are located on the chromosome (Ashiuchi et al., 1999; Nagai et al., 1997). Interestingly, it was found that the inability of non-γ-PGA-producing *Bacillus* strains, which carry the γ-PGA gene cluster, seems to be due to a non-translation of the genes rather than the genes encoding an inactive enzyme complex (Urushibata et al., 2002).

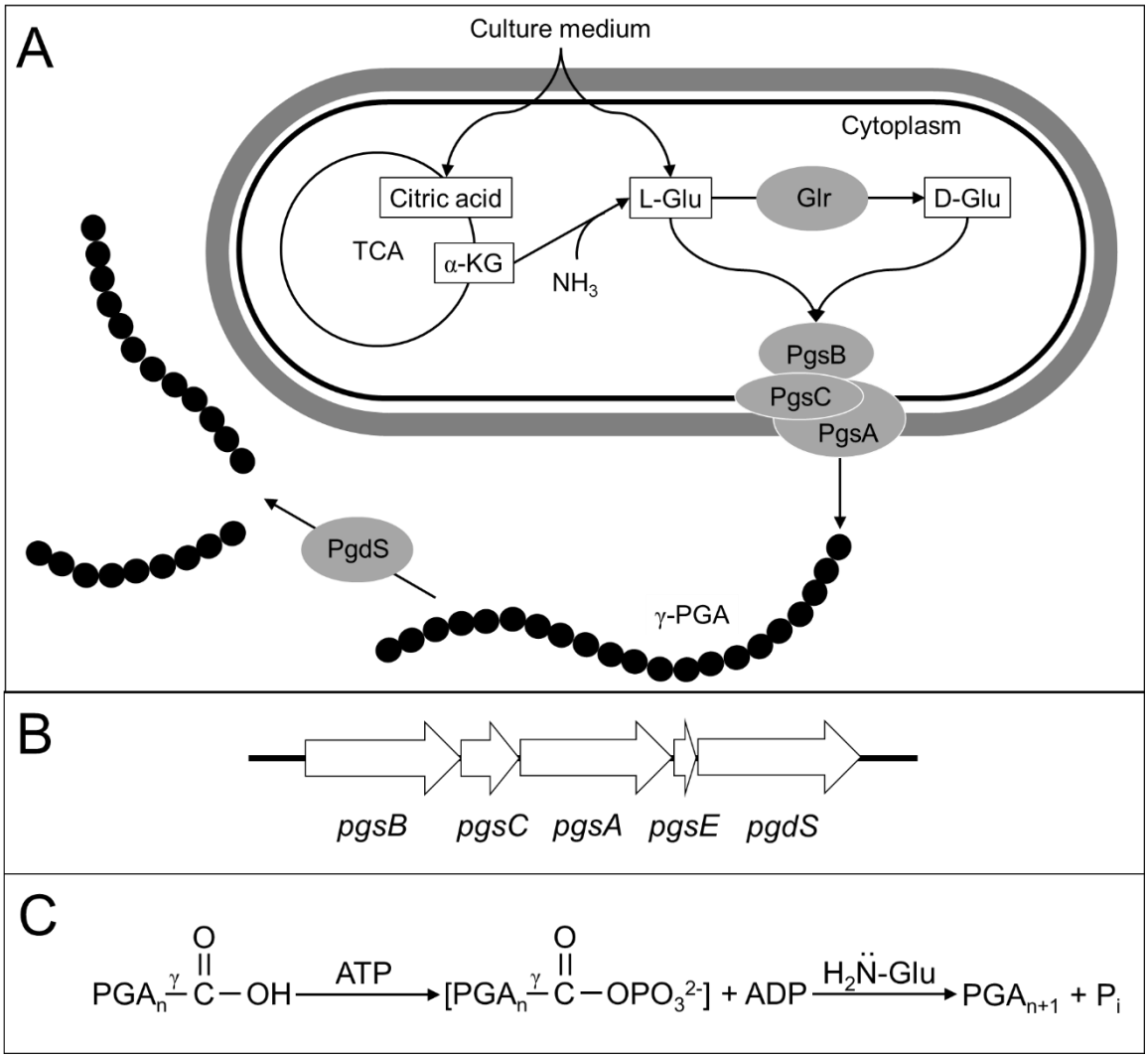

Figure 1-2 Proposed production pathway for γ-PGA in *Bacilli* (A), gene operon for γ-PGA production in *B. subtilis* (B), and proposed elongation mechanism of the γ-PGA synthesis complex (C)

TCA = tricarboxylic acid cycle, α-KG = α-ketoglutarate, L-Gln = L-glutamine, L-Glu = L-glutamic acid, D-Glu = D-glutamic acid, Glr = glutamic acid racemase, Pgs = poly(γ-glutamic acid) synthetase, PgdS = γ-PGA depolymerase, figure adapted from Buescher and Margaritis (2007) **(A)**. *pgsBCA* = subunits of the poly-γ-glutamate synthesis complex, *pgsE* = small protein participating in γ-PGA production, the role is not exactly clear, *pgdS* = γ-DL-glutamyl hydrolase (endo-depolymerase), figure adapted from Ashiuchi (2013) **(B)**. The reaction mechanism for the elongation of the γ-PGA polymer is described in the text, figure adapted from Ashiuchi et al. (2001b) **(C)**.

A synthesis complex with three subunits has been identified as the machinery for γ-PGA production in *B. subtilis*. The proposed production pathway is shown in Fig. 1-2A. The synthesis complex accepts both D- and L-glutamic acid as substrates (Ashiuchi et al., 2001b; Ashiuchi et al., 2004; Ashiuchi et al., 1999). The racemization of L-glutamic acid to

D-glutamic acid is performed by the enzyme glutamic acid racemase (Glr). Glr is a cytosolic enzyme with a high selectivity for glutamic acid and a preference for L-glutamic acid (Ashiuchi et al., 1998). The γ-PGA synthesis complex is membrane-bound, encoded by the operon *pgsBCAE* (*pgs*: *p*oly-γ-*g*lutamate *s*ynthesis; Fig. 1-2B), and so it accepts D- as well as L-glutamic acid as substrate, it has a slight preference for the former (Ashiuchi, 2010; Ashiuchi et al., 2001b; Ashiuchi et al., 2004). The subunits PgsB and PgsC together form the catalytic site of the PgsBCA complex. It is not stereospecific, which is so far unique among transpeptidases. PgsA removes the elongated γ-PGA chain from the catalytic site to allow for the following glutamate monomer to be added and seems to be responsible for the transport of the produced γ-PGA through the membrane (Ashiuchi et al., 2003a; Ashiuchi and Misono, 2002; Ashiuchi et al., 2001b). The exact function of the small protein PgsE is not yet clear, but it is suggested that it has a structural role in γ-PGA synthesis, interacts probably with PgsA, and is not a regulator (Ashiuchi, 2010; Candela et al., 2005). Moreover, a model for the polymerization reaction was proposed, which is depicted in Fig. 1-2C (Ashiuchi et al., 2001b): the free γ-carboxyl group of the γ-PGA-chain is phosphorylated under consumption of ATP, which releases ADP. Subsequently, a γ-peptide bond is formed between the phosphorylated γ-C-atom of the γ-PGA-chain and the α-amino-group of a glutamic acid monomer, whereby P_i is released. Therefore, PgsBCA works in an amide ligase-like manner.

In 2013, the complete genome sequence of *B. licheniformis* ATCC 9945 was published (GenBank entry CP005965) (Rachinger et al., 2013). According to the GenBank entry, the strain ATCC 9945 contains the same γ-PGA synthesis operon as *B. subtilis*. Therefore, results from publications about the γ-PGA synthesis complex of *B. subtilis* and its regulation should be also applicable for *B. licheniformis*.

1.2.3 Degradation of γ-PGA

γ-PGA can be degraded chemically/physically by exposure to extreme pH values and/or high temperatures for a prolonged time (Goto and Kunioka, 1992; Kunioka and Goto, 1994), physically by ultrasonic irradiation (Pérez-Camero et al., 1999), and enzymatically (Birrer et al., 1994; Goto and Kunioka, 1992). Yet, due to its γ-peptide bond, it is stable in the presence of proteases (Kimura and Fujimoto, 2010). γ-PGA degrading enzymes can either act in an endo- or an exo-peptidase manner. γ-PGA producing *B. subtilis* and *B. licheniformis* strains

are reported to secrete endo-γ-glutamyl-peptidases into the medium, which cleave high-molecular weight γ-PGA into smaller fragments with a size of approximately 10^5 Da (Ashiuchi et al., 2003b; Kimura et al., 2004; King et al., 2000). The gene that encodes the endo-γ-glutamyl-peptidase is termed *pgdS* (or alternatively *ywtD; pgd: p*oly-γ-*g*lutamate *d*egradation) and is located directly downstream and in the same orientation as the *pgsBCA* operon, as depicted in Fig. 1-2B (Ashiuchi et al., 2003b). Exo-γ-glutamyl-peptidases (Ggt) further cleave the γ-PGA from the amino-terminal end and release single glutamate monomers (Abe et al., 1997; Kimura et al., 2004). The *ggt* gene is located more distant to the *pgsBCA* operon for chromosome located operons (e.g. in *B. subtilis*) but directly downstream of the operon for the plasmid encoded version in *B. anthracis*. In *B. anthracis*, the enzyme does not hydrolyse γ-PGA but is responsible for anchoring the γ-PGA chains to the cell wall (Candela et al., 2005).

1.2.4 Cultivation conditions for γ-PGA production

For the γ-PGA production with *B. subtilis* and *B. licheniformis*, generally a distinction is made between so-called glutamic acid dependent and independent producers (Ashiuchi, 2013; Bajaj and Singhal, 2011; Buescher and Margaritis, 2007). The former require glutamic acid in the medium to efficiently produce γ-PGA, while the latter are capable of de-novo synthesis of substantial amounts of glutamate. *B. licheniformis* ATCC 9945 (formerly classified as *B. subtilis* ATCC 9945), the strain used in this work, has previously been shown to require glutamic acid in the medium for γ-PGA production (Leonard et al., 1958; Thorne et al., 1954). It is therefore usually classified as a glutamic acid-dependent γ-PGA producer. Interestingly, in the 1990s it was demonstrated that *B. licheniformis* ATCC 9945 also produces γ-PGA in mineral medium without glutamic acid. Here, the achieved γ-PGA concentration was 25 % lower than in the medium containing glutamic acid (Birrer et al., 1994). However, the strain *B. licheniformis* ATCC 9945 is probably the most widely used strain for research purposes (Ashiuchi, 2013; Buescher and Margaritis, 2007).

The applied culture conditions and medium compositions can have a great impact on molecular weight, concentration, and D/L-ratio of the produced γ-PGA. For cultivations, often medium E or variations thereof are used (preparation see Chapter 2.4). Medium E contains substantial amounts of three different carbon sources (glycerol, glutamic acid, and citric acid)

but lacks some trace elements such as zinc and copper and also an inert buffer (Leonard et al., 1958). Only citric acid exhibits some buffering capacity – as long as it is not consumed – due to its third pK_a value at pH 6.4. Medium E is a mineral medium derived from medium C (Leonard et al., 1958). Medium C in turn was developed by Thorne et al. (1954) coming from a version of Sauton's medium (Bovarnick, 1942). Bovarnick's (1942) choice of Sauton's medium "was determined by the desirability of using as simple a medium as possible. Fortunately this species [*Bacillus subtilis*] is generally non-fastidious in its growth requirements, and this particular strain was found to produce peptide on Sauton's medium". The original Sauton's medium contained glycerol, citric acid, and asparagine as well as di-potassium hydrogen phosphate, magnesium sulfate, and ferric ammonium citrate and was used to cultivate *Mycobacterium tuberculosis* (Sauton, 1912). In Bovarnick's version of Sauton's medium, asparagine had been exchanged with glutamic acid and the glycerol concentration reduced to one third (Bovarnick, 1942). Thorne et al. (1954) started from Bovarnick's version of Sauton's medium and developed a medium which is almost identical to the medium E of Leonard et al. (1958). Leonard et al. (1958) simply exchanged the tap water in Thorne's medium with distilled water and added calcium chloride as well as manganese sulfate. Thus, the medium was developed which is predominantly applied until today.

The influence of the different carbon sources in medium E on γ-PGA production was investigated in shake flask cultivations (Birrer et al., 1994). With all three carbon sources (glycerol, glutamic acid, and citric acid), the highest γ-PGA concentration was achieved. It was observed that when γ-PGA production was maximal, citric acid was nearly exhausted, while glycerol and glutamic acid were only utilized by 50 %. When citric acid was omitted from the medium, γ-PGA concentration was lowest, followed by media where glycerol or glutamic acid were omitted. The low obtained γ-PGA concentrations without citric acid or glycerol were probably due to the strong decrease or increase of the pH value, respectively, and not to the lack of the corresponding carbon source itself. Similar observations were already made in the 1950s. Here, however, γ-PGA production was strongly impaired when glutamic acid was omitted from the medium (Thorne et al., 1954). When the effects of glucose and glycerol were explored, medium E formulations with only glucose or glucose/glycerol mixtures all showed better cell growth than standard medium E (which contains glycerol only). γ-PGA concentrations were highest for a 50:50 mixture of glucose and glycerol, followed by a 60:40 mixture and the standard medium E. The lowest γ-PGA concentration was

achieved for glucose only (Gross and Ko, 1998). For some of the medium formulations, low pH values of ~5.5 were observed during cultivation and probably also had an impact on cell growth and product formation. In the same publication, experiments were also carried out in stirred tank reactors at a constant pH value of 6.5 (Gross and Ko, 1998). For the medium formulations tested in these experiments, it was shown that the cells did only grow in the presence of trace amounts (0.5 g L^{-1}) of glutamic acid and citric acid which was attributed to the prevention of salt precipitation. Under these conditions, the fastest cell growth and high γ-PGA production could be obtained with a medium containing only glucose as carbon source.

Already early on, it was shown that by varying the Mn^{2+} concentration in medium E, the ratio of D- to L-glutamic acid could be changed in γ-PGA produced by *B. licheniformis* ATCC 9945. With increasing Mn^{2+} concentrations, the content of D-glutamic acid also increased up to a maximum of approximately 85 % (Leonard et al., 1958). This fact was later reconfirmed (Cromwick and Gross, 1995). For *B. subtilis* chungkookjang, it was found that by varying the NaCl concentration in the medium, the molecular weight of the produced polymer varied (Ashiuchi et al., 2001a). At relatively low NaCl concentrations (≤0.5 %), high molecular weight γ-PGA of more than 2,000 kDa was produced while at very high NaCl concentrations (≥10 %), the molecular weight ranged from 10 to 200 kDa (it was not specified in the publication whether "%" meant "w/v" or "v/v"). For *B. licheniformis* ATCC 9945, the influence of NaCl concentrations in the range of 0 to 4 % (w/v) NaCl on γ-PGA production was investigated and an opposite trend was observed (Birrer et al., 1994). Here, the molecular weight was highest for the highest NaCl concentration and decreased with decreasing NaCl concentration. The γ-PGA concentration, on the other hand, showed a reverse trend and was highest for the reference without any NaCl in the medium and lowest for 4 % NaCl. The differences between these findings are possibly due to the difference in NaCl concentrations which were investigated. In general, it can be concluded that increasing NaCl concentrations induce higher osmolarity and ionic strength in the cultivation medium, and the observations made in these publications demonstrate that these parameters also have an impact on γ-PGA production.

Thorne et al. (1954) observed that filling volume and shaking frequency had a significant effect on γ-PGA production by *B. licheniformis* ATCC 9945 (in the publication still designated as *B. subtilis*). A higher shaking frequency and lower filling volume markedly

increased the yield. Although not explicitly stated in the publication, these findings demonstrate the importance of finding the most suitable level of oxygen supply for γ-PGA production. It should be mentioned that the increase in shaking frequency and decrease in filling volume by Thorne et al. (1954) still must have resulted in a maximum oxygen transfer capacity of only approximately 5 mmol L^{-1} h^{-1} (Seletzky et al., 2007). This means that the cultures were highly oxygen limited and a further increase in shaking frequency or decrease in filling volume would have possibly resulted in even higher γ-PGA yields. Similar investigations were performed for batch fermentations in stirred tank bioreactors (Cromwick et al., 1996). *B. licheniformis* ATCC 9945 was grown in medium E in a 2 L stirred tank with different aerations rates and stirring speeds. Here also, higher aeration rates and stirring speeds increased γ-PGA concentrations. But it was observed as well that even with the highest aeration rate (2 vvm) and stirring speed (800 rpm) tested, the DOT dropped below 1 % during fermentation and, therefore, oxygen limited conditions could not be fully avoided (Cromwick et al., 1996).

Additionally, the authors investigated the effect of different pH values on γ-PGA production and found that a pH value of 6.5, maintained by titration with acid or base, gave the highest γ-PGA concentrations. This pH value proved to be superior to the lower and higher pH values of 5.5, 7.4 or 8.25 and also to an uncontrolled pH value (Cromwick et al., 1996). For shake flask cultivations, a drop from the starting pH value of 7.4 to pH values between ~5.5 to 6.0 was observed in the first 48 h of cultivation. This was attributed to the secretion of acetic acid or acids in general (Birrer et al., 1994; Thorne et al., 1954). If cultivations were continued after the maximal γ-PGA concentration was reached, the pH value increased again up to 8.5 or higher (Thorne et al., 1954).

The selection of topics and references in this subchapter already shows that γ-PGA production by *B. licheniformis* (ATCC 9945) is a complex process and that a multitude of parameters is available for investigation and variation to characterize and optimize growth and product formation. Important parameters certainly include medium composition and, closely related to this, the pH value, while it is furthermore crucial to find an appropriate level of oxygen supply.

1.3 Influence of biopolymer production on fermentations

The production of biopolymers such as γ-PGA increases the viscosity of the fermentation broth during cultivation. The flow behavior of such a fermentation broth is usually pseudo-plastic, meaning that its apparent viscosity decreases with increasing shear rate (Kemblowski and Kristiansen, 1986). Previous studies showed that the broth's viscosity impairs mixing and gas/liquid mass transfer, as well as heat transfer (Büchs et al., 2001; Giese et al., 2014a; Nienow, 1998). For cultivations in shake flasks at elevated viscosity, the system might even get into the so-called "out-of-phase" condition, where the bulk liquid in the shake flask does not follow the rotational force generated by the shaker anymore, but instead remains mainly at the bottom of the shake flask (Büchs et al., 2001). This "out-of-phase" condition leads to a significantly reduced power input into the liquid and, thus, to strongly reduced mixing and mass transfer. Accordingly, its occurrence is usually very unfavorable for fermentations in shake flasks and should be avoided (Giese et al., 2014b). Furthermore, the production of a biopolymer with pseudo-plastic properties also impacts stirred tank bioreactor fermentations. Giese et al. (2014b) showed that the effective shear rates in stirred tank bioreactors are generally lower than in shake flasks (compared at the same specific power input), meaning that the apparent viscosities of pseudo-plastic fermentation broths are even higher in stirred tank bioreactors than those in shake flasks. Hence, biopolymer production massively influences fermentations in shake flasks and stirred tank bioreactors.

If an industrial *Bacillus* production strain not only secretes the target compound, but also produces undesired biopolymers such as γ-PGA, this will considerably interfere with the fermentation process itself and also with the subsequent downstream processing. Although this is frequently noticed (Bulthuis et al., 1988; Giesecke et al., 1991; Herrmann et al., 1997), there are many studies about improving γ-PGA production, but only little work dealing with the prevention of undesired γ-PGA formation (Kambourova et al., 2001; Wilming et al., 2013). As a resulting impairment or failure of fermentation and downstream processes is quite obviously economically unfavorable, it is of great interest to investigate how to prevent the formation of undesirable biopolymers and allow for undisturbed fermentation and downstream processing.

1.4 Online power input measurement in shake flasks

The specific power input per liquid volume is one of the essential parameters for the general characterization of culture conditions in bioreactors for aerobic microorganisms. Moreover, it is important for the scale-up and scale-down of microbial processes. Parameters which directly have an impact on biological cultures such as oxygen supply, carbon dioxide removal, degree of mixing as well as hydromechanical stress or dispersion of an organic liquid phase are all more or less dependent on the specific power input (Büchs et al., 2001). Besides shaking frequency, flask size, and filling volume, the viscosity of the fermentation broth is one of the factors influencing the power input in unbaffled shake flasks (Büchs et al., 2000a, b). As shaking frequency, flask size, and filling volume are usually constant throughout an experiment, a temporal alteration in viscosity is the only variable factor influencing the power input during fermentation in shake flasks. With increasing viscosity, the power input also increases and vice versa. The power input in shake flasks can be determined by measuring the torque of an orbital rotating lab shaker with a torque sensor integrated into the drive of the shaker, a method first introduced by Büchs et al. (2000a). Temporal alterations in viscosity during fermentation processes can be due to filamentous biomass formation or biopolymer production and degradation. The method of online power input measurement to follow biomass or product formation was already successfully applied for different fermentation systems, including the filamentous fungus *Trichoderma reesei*, the xanthan producer *Xanthomonas campestris* (Giese et al., 2014b), and *Azobacter vinelandii* producing alginate (Peña et al., 2007). The method was also used for the optimization of culture conditions in shake flasks to especially prevent unfavorable “out-of-phase” conditions which strongly impair oxygen supply and mixing (Büchs et al., 2001; Lotter and Büchs, 2004; Peter et al., 2004).

1.5 Overview

Bacillus species are used for the production of many commercial products, such as proteases for the detergent industry. This production process can be disturbed by the formation of the biopolymer γ-PGA as a by-product of the protease producing *Bacillus* strain. The biopolymer formation leads to an increase of the viscosity of the fermentation medium, impairing heat and

mass as well as gas transfer in the cultivation system. Therefore, it is important to control and prevent this undesired by-product formation to enable an undisturbed production process. Chapter 3 introduces a protease producing *B. licheniformis* strain which shows the described phenomenon of undesired γ-PGA formation. The influence of nitrogen source and pH value on protease and γ-PGA production was investigated in shake flask and stirred tank reactor cultivations. A simple solution is presented to avoid formation of γ-PGA without impairing the protease production.

Bacillus species, especially *B. subtilis* and *B. licheniformis*, are also used to produce γ-PGA as the main valuable product. A typical *B. licheniformis* production strain for γ-PGA was investigated under standard conditions from the literature, but also with different medium compositions and filling volumes. The results of these experiments are described and discussed in Chapter 4.

Chapter 5 describes the application of power input measurement for shake flask cultivations to follow γ-PGA formation and degradation. For these investigations, the *B. licheniformis* γ-PGA production strain and the protease producing *B. licheniformis* strain with the undesired γ-PGA formation were analyzed and compared regarding their growth and production behavior.

Chapter 2

Materials and methods

2.1 Microbial strains and chemicals

The protease producing *B. licheniformis* strain A1 was kindly provided by Henkel AG & Co. KGaA (Düsseldorf, Germany). The strain *B. licheniformis* A1 secretes γ-PGA as an undesired by-product (Meissner et al., 2015; Wilming et al., 2013). It was stored as glycerol stocks in complex LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 10 g L^{-1} NaCl, pH 7.0) with 30 % (w/v) glycerol at -80 °C. Strain A1 is resistant to kanamycin. Therefore, sterile-filtered kanamycin sulfate was added to the media with a final concentration of 50 mg L^{-1}.

The γ-PGA producing strain *B. licheniformis* ATCC 9945 was acquired from the *American Type Culture Collection* and stored as glycerol stocks in medium E (Leonard et al., 1958) with 30 % (w/v) glycerol at -80 °C.

All chemicals were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Merck KGaA (Darmstadt, Germany), VWR International GmbH (Darmstadt, Germany), or AppliChem GmbH (Darmstadt, Germany), and were of analytical grade.

2.2 Media for protease production of *B. licheniformis* A1

For pre-cultures, a complex medium was used which contained per liter: 25 g veal infusion broth (Difco™ Veal Infusion Broth, N°234420, BD, Heidelberg, Germany) and 5 g yeast extract (Roth, Karlsruhe, Germany). The pH value was checked before autoclaving to be at 7.0

to 7.2. Kanamycin sulfate was added from a sterile-filtered stock solution after autoclaving and prior to cultivation for a final concentration of 50 mg L^{-1}.

The main culture medium V3 (Wilming et al., 2013) for shake flask cultivations and stirred tank bioreactor cultivations with a pH-buffer system contained per liter: 20 g glucose, 1.01 g $MgSO_4 \cdot 7H_2O$, 0.026 g $CaCl_2 \cdot 2H_2O$, 0.05 g $MnCl_2 \cdot 4H_2O$, 15 g $(NH_4)_2SO_4$, 5 mL trace elements stock solution (preparation see below), 0.05 g $FeSO_4 \cdot 7H_2O$, 41.85 g (0.2 M) MOPS acid, 3.4 g K_2HPO_4, and 50 mg kanamycin sulfate. The main culture medium V3 for shake flask cultivations was prepared from distilled water and sterile stock solutions, added in the order of appearance. The pH value of the MOPS acid stock solution was set to 8. Before adding K_2HPO_4, the pH value was checked and, if necessary, re-adjusted to pH 8 with 5 M NaOH. A 1000x trace elements stock solution was prepared, containing per liter: 530 mg $CoCl_2 \cdot 6H_2O$, 260 mg $ZnCl_2$, 10 mg H_3BO_3, 660 mg $NiSO_4 \cdot 6H_2O$, 310 mg $CuSO_4 \cdot 5H_2O$, and 650 mg $Na_2MoO_4 \cdot 2H_2O$. The trace elements stock solution was diluted 1:5 with distilled water to obtain a 200x stock solution, sterile-filtered and used for medium preparation. The $FeSO_4 \cdot 7H_2O$ stock solution was freshly prepared for each experiment and sterilized by filtration. Kanamycin sulfate stock solution was sterile-filtered and stored in aliquots at -20 °C. The MOPS acid stock solution was also sterile filtered. All other stock solutions were sterilized by autoclaving. In some experiments, other nitrogen sources were used instead of $(NH_4)_2SO_4$ in the following concentrations: 12.14 g L^{-1} NH_4Cl, 22.95 g L^{-1} KNO_3, or 19.29 g L^{-1} $NaNO_3$. These concentrations were chosen to give equal molar concentrations of nitrogen compared with $(NH_4)_2SO_4$ as nitrogen source (227 mmol L^{-1} nitrogen).

The main culture medium V3 for stirred tank bioreactor cultivations was prepared differently than that for shake flask cultivations. A basic salt solution containing $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot H_2O$, and $MnCl_2 \cdot 4H_2O$ was sterilized directly in the stirred tank bioreactor. For cultivations with KNO_3 as nitrogen source, KNO_3 was added instead of $(NH_4)_2SO_4$. The pH value of the basic salt solution was set to 7.0 before autoclaving. After sterilization of the stirred tank bioreactor containing the salt solution, stock solutions of the remaining medium components were added aseptically (in order of appearance): glucose, trace elements (preparation see above), $FeSO_4 \cdot 7H_2O$ stock, MOPS acid with a pH value of 8.0, K_2HPO_4, kanamycin sulfate. Before adding K_2HPO_4, the pH value was checked and, if necessary, re-adjusted with 5 M NaOH to pH 8.0 for experiments with MOPS buffer or to pH

7.0 for experiments with pH control. In experiments with pH control, the MOPS acid stock solution was not added, therefore, the volume of the basic salt solution was higher. The final volume in the stirred tank bioreactor was 1.7 L.

2.3 Cultivation conditions for protease production of *B. licheniformis* A1

A two-stage pre-culture was used to inoculate main cultures. The first pre-culture was inoculated from glycerol stock and cultivated overnight under the following conditions: 250 mL shake flasks, filling volume 10 mL, shaking frequency 350 rpm, shaking diameter 50 mm, temperature 37 °C, 4 % (v/v) inoculum. The second pre-culture was inoculated from the first pre-culture to an initial optical density (OD) at 600 nm of OD_{600} = 0.4 and cultivated under the same conditions as the first pre-culture. It was run for about 7 h, when it reached an OD_{600} of ~6.

Online monitoring of both pre-cultures and also of the main culture was realized using an in-house manufactured Respiration Activity Monitoring System (RAMOS) to record the oxygen transfer rate (OTR) (Anderlei and Büchs, 2001; Anderlei et al., 2004). The OTR (mol L^{-1} h^{-1}) was calculated from the measurement of the oxygen partial pressure in the headspace of the RAMOS shake flasks by using Eq. 1.

$$OTR = \frac{n_{O2}}{V_{fl} \cdot t} = \frac{\Delta p_{O2}}{\Delta t} \cdot \frac{V_g}{R \cdot T \cdot V_{fl}} \qquad \text{(Eq. 1)}$$

n_{O2}	moles of oxygen [mol]
V_{fl}	liquid filling volume of the RAMOS shake flask [L]
t	time [h]
Δp_{O2}	difference of oxygen partial pressure [bar]
Δt	duration of the measuring phase [h]
V_g	gas volume of the RAMOS shake flask [L]
R	gas constant [8.314 J mol^{-1} K^{-1}]
T	temperature [K]

For main cultures in shake flasks, a master mix was prepared by inoculating the mineral medium with the second pre-culture. Subsequently, the desired filling volume was transferred to RAMOS flasks and to additional Erlenmeyer flasks with cotton plugs for sampling and off-line analysis. Main cultures were run under the following conditions: 250 mL shake flasks, filling volume 10 (or 25) mL, shaking frequency 350 (or 200) rpm, shaking diameter 50 mm, temperature 37 °C, initial OD_{600} = 0.4. RAMOS flasks and sampling flasks were filled from the same master mix and cultivated in parallel and under identical conditions to guarantee that cultures ran synchronously. Erlenmeyer flasks withdrawn for sampling were not placed back on the shaker.

For main cultures in stirred tank bioreactors, a 3 L BIOSTAT Bplus (Sartorius Stedim Biotech GmbH, Göttingen, Germany) was used, equipped with temperature probe (Pt100), dissolved oxygen tension probe (DOT), and pH probe, a two-stage Rushton turbine with standard geometry (diameter 5.3 cm), and a sampling tube for taking samples with elevated viscosity. Before starting the experiment, the reactor was sterilized at 121 °C and 1 bar overpressure for 21 min. For experiments with pH control, a pH value of 7 was adjusted using 2 M NaOH and 2 M HCl. For all experiments, the cultivation temperature was 37 °C and the filling volume was 1.7 L. The aeration rate was set to 1 vvm. To maintain a DOT above 30 % air saturation, the stirring rate was varried in the range of 100 to 2000 rpm. To avoid foam formation, the silicon-based antifoam Plurafac LF1300 (BASF, Ludwigshafen, Germany) was used in a concentration of 1 mL L^{-1} medium and was added prior to inoculation. For stirred tank bioreactor cultivations, O_2- and CO_2-concentrations were measured using an exhaust gas analyzer (DASGIP GA4, Eppendorf, Wesseling-Berzdorf, Germany). The OTR (mol L^{-1} h^{-1}) was calculated from Eq. 2 (Maier et al., 2001).

$$OTR = \frac{60 \cdot Q_g}{V_m \cdot V_l} \cdot [y_{O2,in} - (\frac{1 - y_{O2,in} - y_{CO2,in}}{1 - y_{O2,out} - y_{CO2,out}}) \cdot y_{O2,out}] \qquad \text{(Eq. 2)}$$

Q_g	aeration rate at standard conditions [L min^{-1}]
V_m	molar volume of an ideal gas at standard conditions [22.414 L mol^{-1}]
V_l	filling volume of the stirred tank bioreactor [L]
$y_{O2,in}$	oxygen mole fraction of the inlet gas [-]
$y_{O2,out}$	oxygen mole fraction of the exhaust gas [-]

$y_{CO2,in}$ carbon dioxide mole fraction of the inlet gas [-]

$y_{CO2,out}$ carbon dioxide mole fraction of the exhaust gas [-]

2.4 Medium for γ-PGA production

For pre- and main cultures the mineral medium E was used (Leonard et al., 1958), which contained per liter: 80 g glycerol, 20 g L-glutamic acid, 12 g citric acid, 7 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.04 g $FeCl_3 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 0.104 g $MnSO_4 \cdot H_2O$. For some experiments, the concentration of citric acid was increased from 12 to 18 g L^{-1} or a trace elements stock solution was added to the medium after autoclaving (preparation see below). Changes in medium composition are indicated in the respective figure captions. The pH value of the medium was set to 7.4 with 5 M NaOH before autoclaving. The medium was sterilized for 20 min at 121 °C and 1 bar overpressure. Prior to inoculation, the pH value was checked and, if necessary, re-adjusted to pH 7.4 with 5 M HCl or NaOH. A 500x trace elements stock solution was prepared and applied for some experiments, containing per liter: 530 mg $CoCl_2 \cdot 6H_2O$, 260 mg $ZnCl_2$, 10 mg H_3BO_3, 660 mg $NiSO_4 \cdot 6H_2O$, 310 mg $CuSO_4 \cdot 5H_2O$, and 650 mg $Na_2MoO_4 \cdot 2H_2O$. The trace elements stock solution was diluted 1:5 with distilled water to obtain a 100x stock solution, sterile filtered and used for medium preparation (10 mL trace elements stock solution per liter medium).

2.5 Cultivation conditions for γ-PGA production

For the experiments in Chapter 4, main cultures were directly inoculated by glycerol stocks with 0.4 % (v/v) (Birrer et al., 1994). For the experiments in Chapter 5, a one-stage pre-culture was used to inoculate main cultures to improve the reproducibility of growth and product formation of the main cultures and to be able to use one batch of glycerol stocks for a long series of experiments. The pre-culture was inoculated with 0.4 % (v/v) from glycerol stock and cultivated for 16 h (strain ATCC 9945) or 24 h (strain A1). For main cultures, a master mix was prepared by inoculating the medium either with the glycerol stock (0.4 % v/v) or with the pre-culture to obtain an initial $OD_0 = 0.05$. Then, the desired filling volume was transferred to modified Erlenmeyer (RAMOS) flasks and to normal Erlenmeyer flasks with cotton plugs for sampling and online power input measurement. Pre-culture and main culture (with one

exemption) were both cultivated under the following conditions: 500 mL (exemption: 250 mL) shake flasks, filling volume 100 mL (exemption: 10, 15, 30, 61 mL), shaking frequency 250 rpm, shaking diameter 50 mm, temperature 37 °C. Online monitoring of the OTR of pre-culture and main culture was also realized using the Respiration Activity Monitoring System (RAMOS) (Anderlei and Büchs, 2001; Anderlei et al., 2004). RAMOS flasks, sampling flasks, and flasks for the online power input measurement were always filled from the same master mix and cultivated in parallel and under identical conditions to guarantee that cultures ran synchronously. Erlenmeyer flasks withdrawn for sampling were not placed back on the shaker.

2.6 Online power input measurement

The online measurement of power input was performed with a special lab shaker with a torque sensor integrated into the drive of the shaker, as described by Büchs (Büchs et al., 2000a, b) and Giese (Giese et al., 2014b). The shaker is a modified shaker from Kühner AG (Type LS-X, Birsfelden, Switzerland). A motor drive (Visco-Pakt rheo-110, Hitec Zang GmbH, Herzogenrath, Germany) enables the adjustment and control of the shaking frequency at the same time as well as automated and continuous torque readout (sampling rate: 5 s^{-1}). Reference measurements were conducted in order to determine and consider the influence of friction losses due to mechanical friction in the ball bearing of the shaker and aerodynamic resistance of the shake flasks. For these reference measurements, the liquid was replaced by a solid mass with the same weight as the liquid. Therefore, the pure torque values, resulting from the friction between liquid and shake flask wall, could be determined. The online volumetric power input P/V_L (W m^{-3}) can be calculated with Eq. 3 (Klöckner et al., 2012):

$$\frac{P}{V_L} = 2 \cdot \pi \cdot n \cdot \frac{M}{V_L} = 2 \cdot \pi \cdot n \cdot \frac{M_{liquid} - M_{solid}}{V_L} \quad (3)$$

n	shaking frequency [s^{-1}]
M	online measured torque signal [N m]
V_L	filling volume of all shake flasks [m^3]
M_{liquid}	online measured torque signal of the shake flasks filled with liquid fermentation medium [N m]

M_{solid} online measured torque signal of the empty shake flasks plus the solid mass of the same weight as the liquid [N m]

Additionally, the liquid loss due to evaporation from the shake flasks during the experiments was determined and the reduction in filling volume was considered when calculating the volumetric power input. As an example, the liquid loss for 500 mL shake flasks with 100 mL filling volume, at a shaking frequency of 250 rpm, a shaking diameter of 50 mm, and a temperature of 37 °C was 9 % after 168 h (7 days), 13 % after 240 h (10 days), and 17 % after 336 h (14 days). These are exemplary values from certain time points to demonstrate the order of magnitude of the liquid loss. The liquid loss was determined at each sampling time point for all flasks withdrawn for sampling.

To calculate the volumetric power input from the effective viscosity determined offline, as it was done for the cultivation of strain ATCC 9945 under standard conditions (Fig. 5-7), the density of the fermentation broth has to be known or estimated. To determine whether the density of the broth changes due to γ-PGA formation, the density of a cultivation under standard conditions was measured at room temperature (20 °C) after 25, 48, and 72 h, using a 50 mL graduated flask and a balance. The density at all three time points was in the range of 1025 to 1031 kg m^{-3}, and therefore close to the 1000 kg m^{-3}, which are usually assumed, if no measured data are available. For an effective viscosity of 100 mPa s under the cultivation conditions listed in the paragraph above, the deviation between a density of 1000 and 1031 kg m^{-3} for the calculation of the volumetric power input is only 1.4 % (in relation to the power input calculated with a density of 1000 kg m^{-3}). Therefore, the slight difference in density was considered negligible, and a density of 1000 kg m^{-3} was used for all calculations.

2.7 Offline analysis

2.7.1 Biomass

Biomass was determined by optical density measurement at 600 nm (Genesys 20 Visible Spectrophotometer, Thermo Scientific, Waltham, U.S.A.). Samples were diluted appropriately (between 1:2 and 1:50, to remain below an extinction of 0.3) with 0.9 % NaCl solution to not exceed the linear measuring range of the photometer. Cell dry weight (CDW) was also

determined but was found to be strongly biased by γ-PGA formation. Therefore, only data for optical density measurement are shown.

For cultivations with *B. licheniformis* A1 for protease production (Chapter 3), a correlation between optical density and cell dry weight was established for approximate cell dry weight quantification. For this correlation, the data from the experiments with nitrate as nitrogen source and under glucose and oxygen unlimited conditions were used. The following correlation was obtained:

$$CDW\ [g\ L^{-1}] = 0.676\ \cdot OD_{600} + 1.274 \qquad \text{(Eq. 4)}$$

2.7.2 Viscosity

The viscosity of the fermentation broth was measured with a Physica MCR301 rheometer (Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) in a range of shear rates between 10 s^{-1} and 2000 s^{-1}. The rheometer was equipped with a cone-plate measuring system from Anton Paar (cone CP50-0.5/TG with cone diameter 49.945 mm, cone angle 0.467°, and cone truncation 54 µm; plate P-PTD200/TG+H-PTD200). Data analysis was performed with the software RheoPlus/32 V3.40 (Anton Paar). 480 µL of fresh, untreated sample were used for viscosity measurements at 37 °C. As γ-PGA solutions exhibit pseudo-plastic properties, the effective viscosity depends on the shear rate which is present in the shake flask at the time point of sampling. The viscosity values presented in Chapter 3 are for a distinct shear rate of $\gamma = 300\ s^{-1}$. For the viscosity values in Chapters 4 and 5, the effective shear rate and effective viscosity were calculated for each viscosity measurement of each sample according to the equation recently published by Giese et al. (2014b). Viscosity measurements were chosen as an additional, simple, and general method to follow γ-PGA formation and degradation during the cultivation besides gel permeation chromatography (GPC), which was only used for the experiments presented in Chapter 5.

2.7.3 Protease activity

The protease activity displayed in Chapter 3 was measured according to the method developed by DelMar et al. (1979). The artificial substrate Suc-AAPF-pNA (N-Succinyl-L-Alanyl-L-Analyl-L-Prolyl-L-Phenylalanin-para-Nitroaniline) is cleaved by the protease, releasing the chromophore *p*-nitroaniline (pNA). This leads to an increase of absorption at 405 nm. The slope of the increase of absorption is proportional to the protease activity. The measurements were performed with cell free supernatant. The supernatant was appropriately (1:20 to 1:1280) diluted with 0.1 M Tris HCl buffer, pH 8.6, 0.1 % (w/v) Brij 35. A stock solution of the substrate Suc-AAPF-pNA (Bachem AG, Bubendorf, Switzerland) was prepared by dissolving 70 mg mL^{-1} in dimethyl sulfoxide and was stored at -20 °C. For the assay, the stock solution was diluted 1:20 with 0.1 M Tris HCl buffer, pH 8.6, 0.1 % (w/v) Brij 35. The assays were performed in transparent 96-well microtiter plates (Rotilabo microtest plates, F-profile (flat bottom), Roth, N° 9293.1) in a microtiter plate reader (Synergy 4, BioTek, Winooski, VT, USA). The assays were conducted at a temperature of 30 °C for 5 min. The kinetic reads started with the addition of the substrate to the samples in the microtiter plate. The volume of the diluted samples was 150 µL to which 50 µL substrate solution was added. Samples were measured as duplicates. As blank, 150 µL of the Tris HCl buffer was used. The protease activity was calculated from the change of absorption at 405 nm with an extinction coefficient of pNA of 8.48 cm^2 μmol^{-1}. Moreover, the maximum specific protease formation rate was calculated using the data of the measured protease activity and the established OD-CDW correlation (Eq. 4).

2.7.4 Poly(γ-glutamic acid) concentration and molecular weight

Concentration and mass average molecular weight (M_w) of γ-PGA was determined by gel permeation chromatography (GPC). The GPC was equipped with a TSKgel GMPWxl column (300 x 7.8 mm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a TSKgel PWxl guard column (40 x 6 mm, Tosoh Bioscience GmbH, Stuttgart, Germany). The column was eluted with 30 mM NaCl at 40 °C at a flow rate of 1.0 mL min^{-1}. Peaks were detected with a Shodex RI-71 refractometer (Showa Denko Europe, Germany). Data analysis was performed with the software Chromeleon 6.2 (Dionex, Germany). Poly(ethylene oxide) standards with a peak molecular weight M_p in the range of 56 to 1015 kDa (PSS Polymer Standards, Service GmbH,

Mainz, Germany) were used to create a calibration curve for molecular weight. γ-PGA sodium salt (cosmetic grade; kindly provided by Henkel AG & Co. KGaA, Düsseldorf, Germany) was used to create a calibration curve to determine concentrations. Culture broth was diluted 1:50 with distilled water and filtered with 0.2 µm filters (Rotilabo syringe filters Mini-Tip, cellulose acetate membrane, N° PP52.1, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). 20 µL of the filtrated samples were injected.

2.7.5 Glucose and overflow metabolites

Concentrations of glycerol, citric acid, and overflow metabolites (acetate, acetoin, 2,3-butanediol) were determined from the supernatant of centrifuged culture broth by HPLC. If necessary, the supernatant was diluted with distilled water (1:4 or 1:10). Samples were sterile filtered with 0.2 µm filters (Rotilabo syringe filters Mini-Tip, cellulose acetate membrane, N° PP52.1, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The HPLC (Ultimate 3000, Dionex, USA) was equipped with an Organic Acid-Resin-Column (250 x 8 mm, CS-Chromatographie Service GmbH, Langerwehe, Germany) and an Organic Acid-Resin-Precolumn (40 x 8 mm, CS-Chromatographie Service GmbH, Langerwehe, Germany). The column was eluted with 5 mM H_2SO_4 at 60 °C at a flow rate of 0.8 mL min^{-1}. Peaks were detected with a Shodex RI-101 refractometer (Showa Denko Europe, Germany). Data analysis was performed with the software Chromeleon 6.2 (Dionex, Germany).

2.7.6 L-glutamic acid

Concentration of L-glutamic acid was measured from the supernatant of centrifuged culture broth with a commercially available enzyme test kit (L-Glutamic Acid Assay Kit, Megazyme International Ireland, Bray, Co. Wicklow, Ireland). Samples were treated and diluted as indicated in the manual. The assays were performed in transparent 96-well microtiter plates (Rotilabo microtest plates, F-profile (flat bottom), N° 9293.1, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in a microtiter plate reader (Synergy 4, BioTek, Winooski, VT, U.S.A.). The assays were conducted at a temperature of 25 °C. Samples were measured in triplicate.

2.7.7 Ammonium, nitrate, and phosphate

Concentrations of ammonium, nitrate, and phosphate ions were determined photometrically from the supernatant of centrifuged culture broth using the commercially available Spectroquant® test kits from Merck KGaA (Darmstadt, Germany):

- Ammonium: 2.0-150 mg L^{-1} NH_4-N/2.6-193 mg L^{-1} NH_4^+, N°1006830001
- Nitrate: 0.10-25.0 mg L^{-1} NO_3-N/0.4-110.7 mg L^{-1} NO_3^-, N° 1097130001
- Phosphate: 1.0-100.0 mg L^{-1} PO_4-P/3-307 mg L^{-1} PO_4^{3-}/2-229 mg L^{-1} P_2O_5, N°1007980001

The assays were conducted according to the manufacturer's instructions. Samples were appropriately diluted with distilled water to be in the measuring range of the assay.

Chapter 3

Influence of nitrogen source and pH value on undesired poly(γ-glutamic acid) formation of a protease producing *B. licheniformis* strain

The results in this chapter demonstrate the phenomenon of undesired γ-PGA production of an industrial protease producing *B. licheniformis* strain (A1) and present a simple solution for this problem, which can be applied for shake flask and stirred tank bioreactor cultivations. It is shown how an exchange of the nitrogen source in a defined mineral medium prevents undesirable γ-PGA formation without influencing the protease formation. Additionally, a first and simple attempt is made to explain why the exchange of nitrogen source is able to prevent γ-PGA formation. Since the objective was to present a method to prevent undesired γ-PGA formation by *Bacillus licheniformis* strains used for the production of industrial enzymes, the exact determination of γ-PGA concentrations was not a focus of these experiments. Therefore, the simple means of viscosity measurements with a rheometer was chosen to easily follow γ-PGA production during fermentations compared to more complicated and time-consuming analytical techniques like gel permeation chromatography. All results presented in this chapter are published (Meissner et al., 2015) and are reprinted with permission of Springer. Some of the results in this chapter were obtained by the work of Timo Wengeler (research internship) and Kira Kauffmann (bachelor thesis).

3.1 Influence of ammonium and nitrate on viscosity build-up in shake flask fermentations

In the experiments described and discussed in this chapter, the influence of ammonium and nitrate as nitrogen sources on viscosity build-up of a protease producing *B. licheniformis* strain (A1) in shake flask fermentations was investigated. Figure 3-1 displays the results of these shake flask fermentations. In the mineral medium used for protease production, ammonium sulfate is applied as nitrogen source (Figs. 3-1A-C). Figure 3-1A displays oxygen transfer rate (OTR) and optical density (OD_{600}) of the fermentation as parameters for cell growth. After a very short lag phase of about 2 h, both OTR and OD_{600} increased exponentially until 12 h of cultivation, indicating unlimited growth of *B. licheniformis* A1. The OTR reached a maximum of 56 mmol L^{-1} h^{-1} before declining sharply, thereby notifying the depletion of glucose (Fig. 3-1C). The maximum OD_{600} of 11 coincided with the maximum OTR. Thereafter, the OD_{600} decreased due to morphological changes of the *B. licheniformis* cells, which went from large to smaller rods and even to coccus-shaped cells (visible with microscopy, pictures not shown). The OD_{600} probably also decreased due to beginning of autolysis of the cells, since fewer cells were visible in the microscopic pictures.

For shake flask cultivations, MOPS buffer was applied for pH control. During cultivation, the pH value decreased from its initial value of pH 8.0 to a minimum of pH 6.6 (Fig. 3-1B). The decrease was due to ammonium consumption and acetate production, a by-product from overflow metabolism. Ammonium decreased to a minimum of 120 mmol L^{-1} while acetate was formed in concentrations of up to 0.85 g L^{-1} (Fig. 3-1C). Minimal pH value and maximum OTR corresponded. Starting with the depletion of glucose and the sharp decrease of OTR, the pH value increased again up to pH 7.4 until the end of the fermentation. One reason for this increase was the consumption of acetate. Under the applied fermentation conditions, an increase of viscosity could be observed during the cultivation process up to 32 mPa s, due to the production of a viscosity elevating by-product (Fig. 3-1B). Since various *Bacillus* species are known to produce the biopolymer γ-PGA (Bajaj and Singhal, 2011; Buescher and Margaritis, 2007), it was suspected that the viscosity elevating by-product is indeed γ-PGA, undesirably formed by the protease producing *B. licheniformis* strain A1.

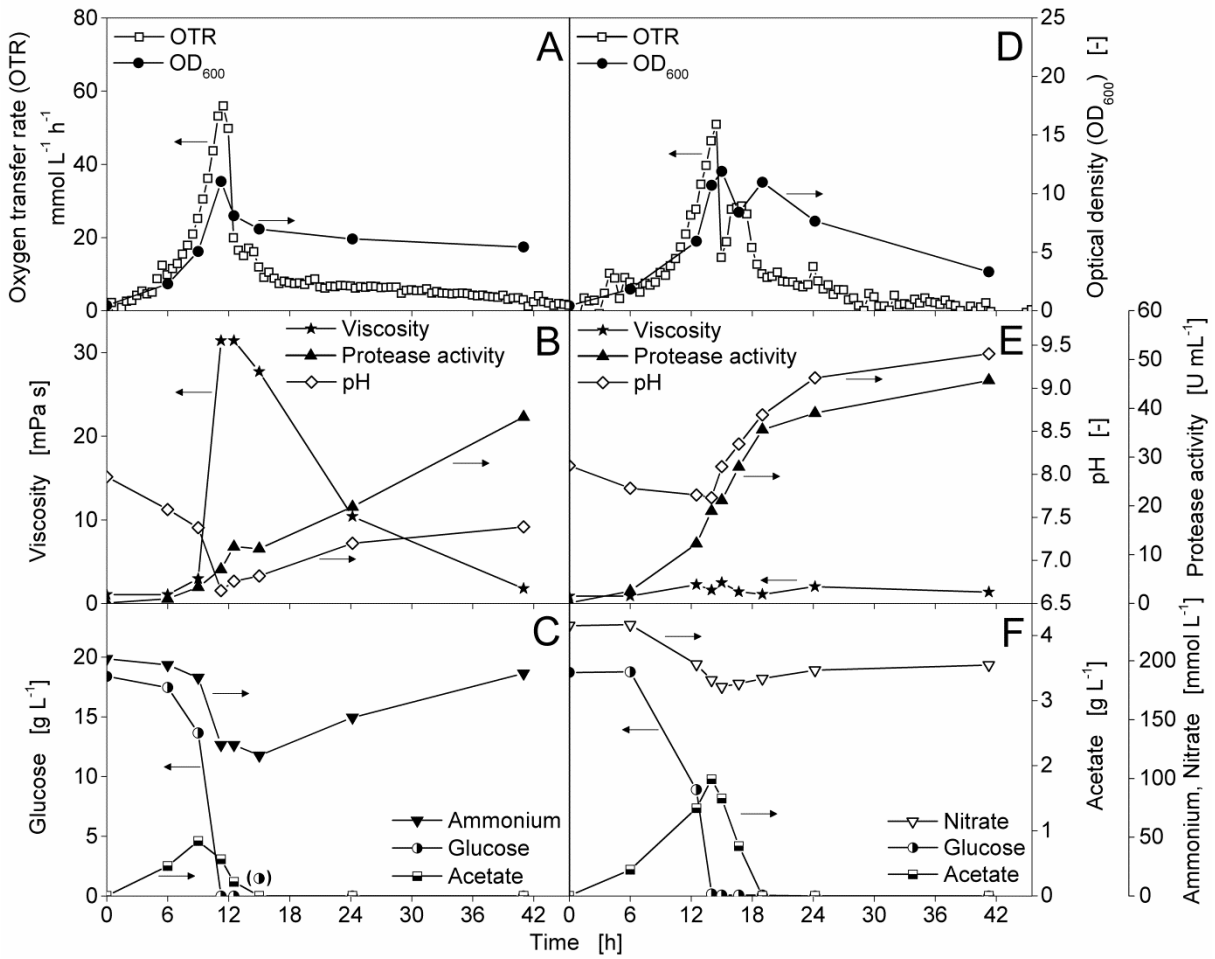

Figure 3-1 Impact of nitrogen source on viscosity build-up due to poly(γ-glutamic acid) formation during fermentation in a mineral medium

Comparison of *B. licheniformis* A1 cultivations in shake flasks with medium containing ammonium **(A-C)** or nitrate **(D-F)** as nitrogen source. Oxygen transfer rate (OTR) and optical density (OD_{600}) are shown in **(A)** and **(D)**. Viscosity at a shear rate of 300 s^{-1}, protease activity, and pH are presented in **(B)** and **(E)**. Glucose, acetate, and ammonium or nitrate concentrations are shown in **(C)** and **(F)**. Acetoin and 2,3-butanediol were not detected in any of the samples. Initial values: OD_{600} = 0.4, pH = 8.0, 20 g L^{-1} glucose, 15 g L^{-1} $(NH_4)_2SO_4$ or 22.95 g L^{-1} KNO_3, 0.2 M MOPS buffer. Cultivation conditions: T = 37 °C, 250 mL shake flasks, filling volume 10 mL, shaking frequency 350 rpm, shaking diameter 50 mm.

The elevated viscosity of the fermentation broth heavily interfered with sample preparation methods like centrifugation or filtration, which are also classical downstream processing techniques. The maximum viscosity coincided with the maximum OTR. The viscosity decreased after that, and at the end of the fermentation the initial viscosity of ~1 mPa s was restored. Similar to the by-product acetate, the viscosity elevating by-product γ-PGA is first produced during cultivation and later on apparently degraded and consumed. Two γ-PGA

degrading enzymes from γ-PGA producing *B. subtilis* strains have been identified, one acting in an endopeptidase-like fashion, cleaving the polymer into smaller oligomers (Ashiuchi et al., 2003b). These oligomers are further cleaved by an exopeptidase into single glutamic acid monomers (Abe et al., 1997). The consumption of glutamic acid released from the degradation of γ-PGA and the accompanying release of ammonium is another reason, besides the uptake of acetate, for the increase of the pH value after glucose depletion. The released ammonium increased the concentration in the fermentation broth from the minimum of 120 mmol L^{-1} to 190 mmol L^{-1} until the end of the fermentation.

Protease activity was rather low during the exponential growth phase up to 12 h (Fig. 3-1B). The majority of protease was produced during the growth on acetate and γ-PGA and in the stationary phase, respectively. The final obtained protease activity was 38 U mL^{-1} and the maximum protease formation rate was 262 U h^{-1} g^{-1} CDW at 11.7 h of cultivation.

The influence of an alternative nitrogen source on protease and γ-PGA formation was investigated. Potassium nitrate was chosen as an alternative to ammonium sulfate. For comparability, the same molar concentration of nitrogen was applied. All other medium compounds and cultivation conditions were identical. Results from this experiment are shown in Figs. 3-1D-F. As for the reference cultivation (Figs. 3-1A-C), OTR and OD_{600} showed exponential increases indicating unlimited growth conditions. The OTR dropped when glucose was depleted, but showed – in contrast to the reference – a distinctive second peak, although smaller than the first peak. This second growth phase is due to the consumption of previously produced acetate. With nitrate as nitrogen source, approximately twice as much acetate (1.8 g L^{-1}) was produced as with ammonium. The pH value also decreased from the initial value of pH 8.0, but much less than in the reference cultivation and reached as a minimum only pH 7.7. This minimum corresponded with the depletion of glucose and the maximum acetate concentration. After reaching its minimum, the pH value increased, but much more than in the reference cultivation, and displayed a final value of 9.4. The slight decrease at the beginning can be attributed to the formation of acetate, while the strong increase results from the consumption of acetate and nitrate. Contrary to ammonium, which leads to a decrease in pH value when consumed, nitrate consumption increases the pH value. The nitrate concentration, starting at 230 mmol L^{-1}, reached a minimum of 178 mmol L^{-1} after 15 h (Fig. 3-1D). From there, the nitrate concentration remained nearly constant until the end of the cultivation.

Interestingly, when nitrate was applied as nitrogen source, no viscosity build-up was observed, indicating that γ-PGA is not formed under these conditions (Fig. 3-1E). Protease production was not much altered: a slightly higher protease activity of 46 U mL^{-1} was observed at the end of the fermentation (Fig. 3-1E). However, the maximum protease formation rate was with 472 U h^{-1} g^{-1} CDW at 12.5 h of cultivation nearly twice as high as in the reference cultivation with ammonium.

To further verify the influence of ammonium and nitrate on undesired γ-PGA formation, also ammonium chloride and sodium nitrate were investigated as nitrogen sources for the protease producing *B. licheniformis* strain A1 and compared with ammonium sulfate and potassium nitrate. OTR curves and results of viscosity measurements are depicted in Fig. 3-2. The oxygen transfer rates of all four approaches show an exponential increase in the first 13 h of cultivation (Fig. 3-2A). Maximum OTRs between 48 and 61 mmol L^{-1} h^{-1} were reached. A steep decrease in the OTR curves indicates a depletion of the carbon source glucose. For the cultures with nitrate as nitrogen source, a distinct second OTR peak is visible. This peak can be attributed to the consumption of acetate which was formed as an overflow metabolite in the first growth phase. The cultures with ammonium do not show a clear second peak in the OTR curve but rather a long plateau at an OTR of 5 mmol L^{-1} h^{-1} until the end of the cultivation.

Figure 3-2B displays the results of a viscosity measurement of all four cultures after 13 h of cultivation. The sample was taken at the time point of maximum OTR, easily detectable from the data of the RAMOS online measuring technique. It indicates the time point of the maximum viscosity (compare Figs. 3-1A and B). For both cultures with ammonium as nitrogen source, viscosities of more than 25 mPa s were obtained. In comparison, the cultures with nitrate had viscosities of approximately 2 mPa s. Therefore, it can be concluded that the influence on undesired γ-PGA formation derives from the ammonium and nitrate ions themselves and not from the availability or absence of any counter ions such as sulfate, chloride, potassium, or sodium.

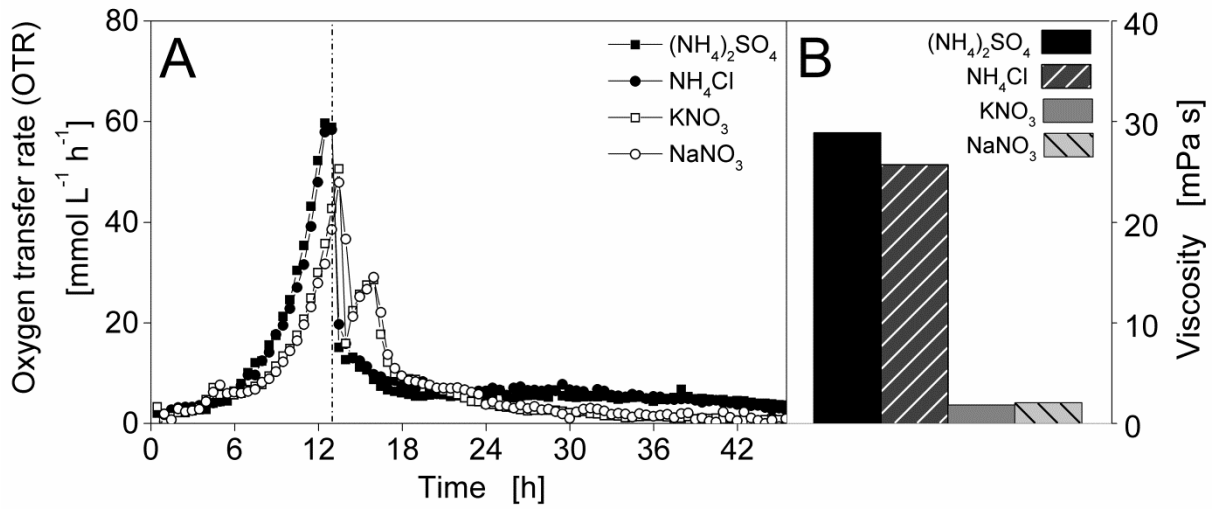

Figure 3-2 Impact of two ammonium and two nitrate salts on viscosity build-up due to poly(γ-glutamic acid) formation during fermentation in a mineral medium

Comparison of *B. licheniformis* A1 cultivations in shake flasks with media containing ammonium sulfate, ammonium chloride, potassium nitrate, or sodium nitrate as nitrogen source. Oxygen transfer rates (OTR) are shown in **(A)**. Viscosities at a shear rate of 300 s^{-1} are presented in **(B)**. Viscosities were measured after 13 h (the time point of sampling is indicated by the dotted line in **(A)**). Initial values: OD_{600} = 0.4, pH = 8.0, 20 g L^{-1} glucose, 15 g L^{-1} $(NH_4)_2SO_4$ or 12.14 g L^{-1} NH_4Cl or 22.95 g L^{-1} KNO_3 or 19.29 g L^{-1} $NaNO_3$ (equals 227 mmol L^{-1} nitrogen in each case), 0.2 M MOPS buffer. Cultivation conditions: T = 37 °C, 250 mL shake flasks, filling volume 10 mL, shaking frequency 350 rpm, shaking diameter 50 mm.

The prevention of undesired γ-PGA by applying nitrate as the sole nitrogen source can probably be explained by taking a look at the nitrogen metabolism of *Bacillus*, which is schematically shown in Fig. 3-3. *B. licheniformis* has two pathways for ammonium assimilation via glutamate synthesis: the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway and the reductive amination of α-ketoglutarate to glutamate by glutamate dehydrogenase (GDH). Both reactions consume one molecule of ammonium and NAD(P)H (Fig. 3-3) (Bernlohr et al., 1984; Schreier, 1993). At high ammonium concentrations, the levels of GDH and GOGAT are high, respectively (Bernlohr et al., 1984). Apparently both enzymes are used for glutamate synthesis. For GDH, which is able to catalyze either the anabolic or the catabolic reaction, also the role of a "regulatory valve" is proposed, which regulates the intracellular glutamate pool (Meers and Pedersen, 1972). Under conditions of low ammonium concentrations, probably only the GS-GOGAT pathway is used for the synthesis of glutamate, since GDH has a relatively low affinity for ammonium (Schreier, 1993).

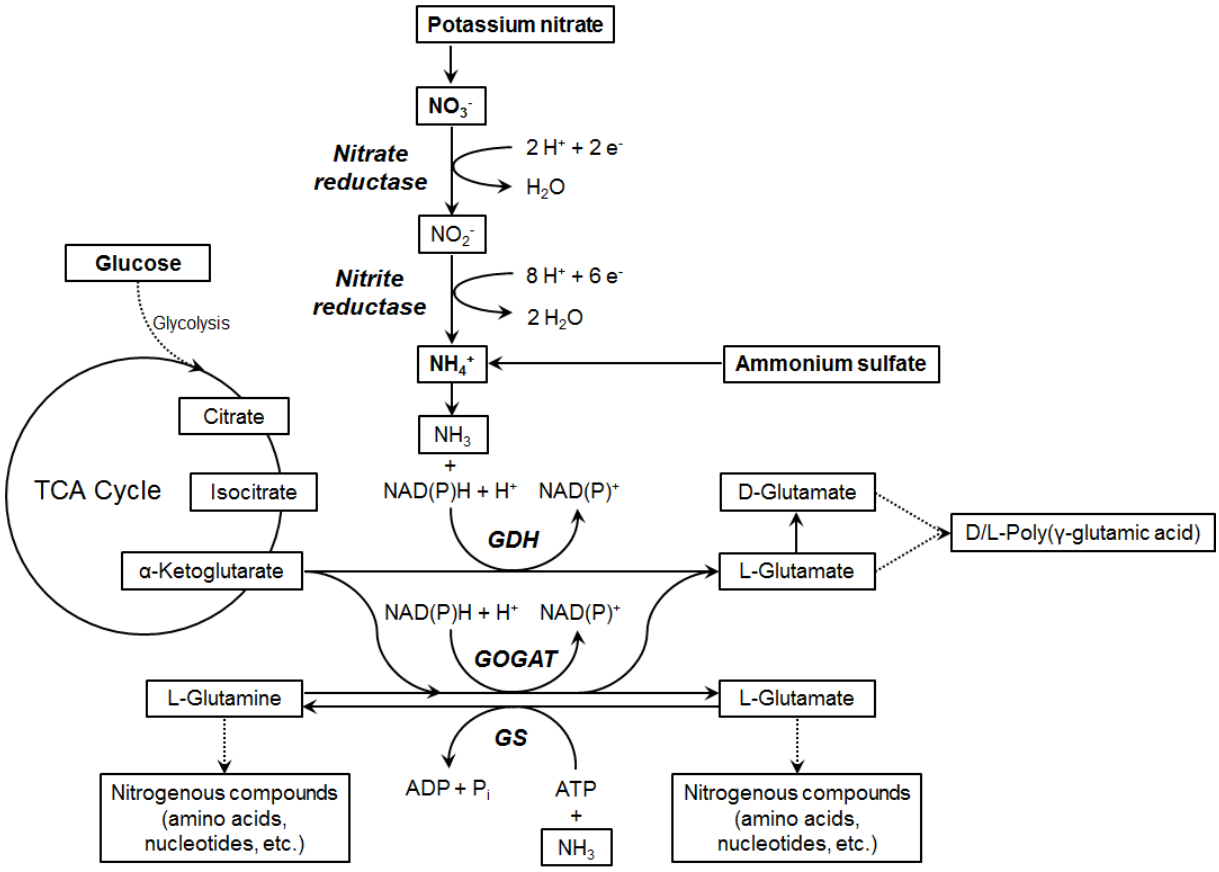

Figure 3-3 Pathways of ammonium and nitrate utilization as nitrogen sources in *B. licheniformis*
GDH: glutamate dehydrogenase, GS: glutamine synthetase, GOGAT: glutamate synthase.

Since the original protease production process in shake flasks is conducted in glucose batch mode with ammonium sulfate as nitrogen source, both α-ketoglutarate and ammonium probably were available in excess. From α-ketoglutarate and ammonium, glutamate is formed in a single step. With excess L-glutamate, *Bacillus* can easily produce γ-PGA (Fig. 3-3). Ammonium as nitrogen source is preferred over nitrate, since it is more easily assimilated. Nitrate bears the disadvantage that it first has to be reduced to ammonium before it can be incorporated into organic nitrogenous compounds such as glutamate and glutamine. This is performed by the enzymes nitrate and nitrite reductase (Nakano et al., 1995; Ogawa et al., 1995). For their reductase activity, both enzymes require several mol NAD(P)H per mol nitrate as electron and proton donators (Richardson et al., 2001). Due to this higher energy cost, nitrate will be reduced to ammonium only in necessary amounts. Therefore, ammonium will most probably never be available in excess under these conditions.

3.2 Influence of pH value on viscosity build-up in stirred tank bioreactor fermentations

In shake flask cultivations with a buffer system, the application of nitrate as nitrogen source leads to a different pH profile over time compared to ammonium. In contrast, production processes in stirred tank bioreactors are usually performed with pH titration instead of applying buffer systems for pH control which leads to a constant pH throughout the fermentation. To study the influence of a changing or a constant pH value on the formation of the by-product γ-PGA, cultivations in stirred tank bioreactors were conducted. Thereby, it should also be investigated whether the chemical nature of the nitrogen source or the pH profile is responsible for production or prevention of undesired γ-PGA.

Figure 3-4 shows the results of two stirred tank bioreactor fermentations with ammonium sulfate as nitrogen source. The significant difference between them is the pH control: Figs. 3-4A-C display a fermentation buffered with MOPS acid and Figs. 3-4D-F show a fermentation with pH titration. The fermentation with buffer was conducted to demonstrate the transferability of the results from 250 mL shake flasks to a 3 L stirred tank bioreactor. The stirred tank bioreactor fermentation with MOPS buffer also had an initial pH value of 8.0, just like the reference in shake flasks (compare Fig. 3-1B). The lag phase in the fermenter cultivation was slightly longer than in the respective reference shake flask cultivation, but the progression curves of the different parameters OTR, OD_{600}, pH, viscosity, protease, glucose, acetate, and ammonium concentrations are similar. In this fermentation, more biomass was formed, since the OD_{600} reached a maximum of 15 compared to 11 in the reference process. Slightly more acetate was formed as well. In contrast, viscosity was lower than in the reference process and reached a maximum of only 12 mPa s compared to 34 mPa s. The lower viscosity can be attributed to the higher biomass and acetate formation observed. The carbon source directed to biomass and acetate formation was, therefore, not available for biopolymer production. For this fermentation, it was observed that prior to inoculation some precipitates had formed in the medium. When the formation of precipitates was observed in other experiments, often the viscosity was lower, probably because one or more nutrients necessary for the formation of γ-PGA were partially removed from the medium by the precipitation.

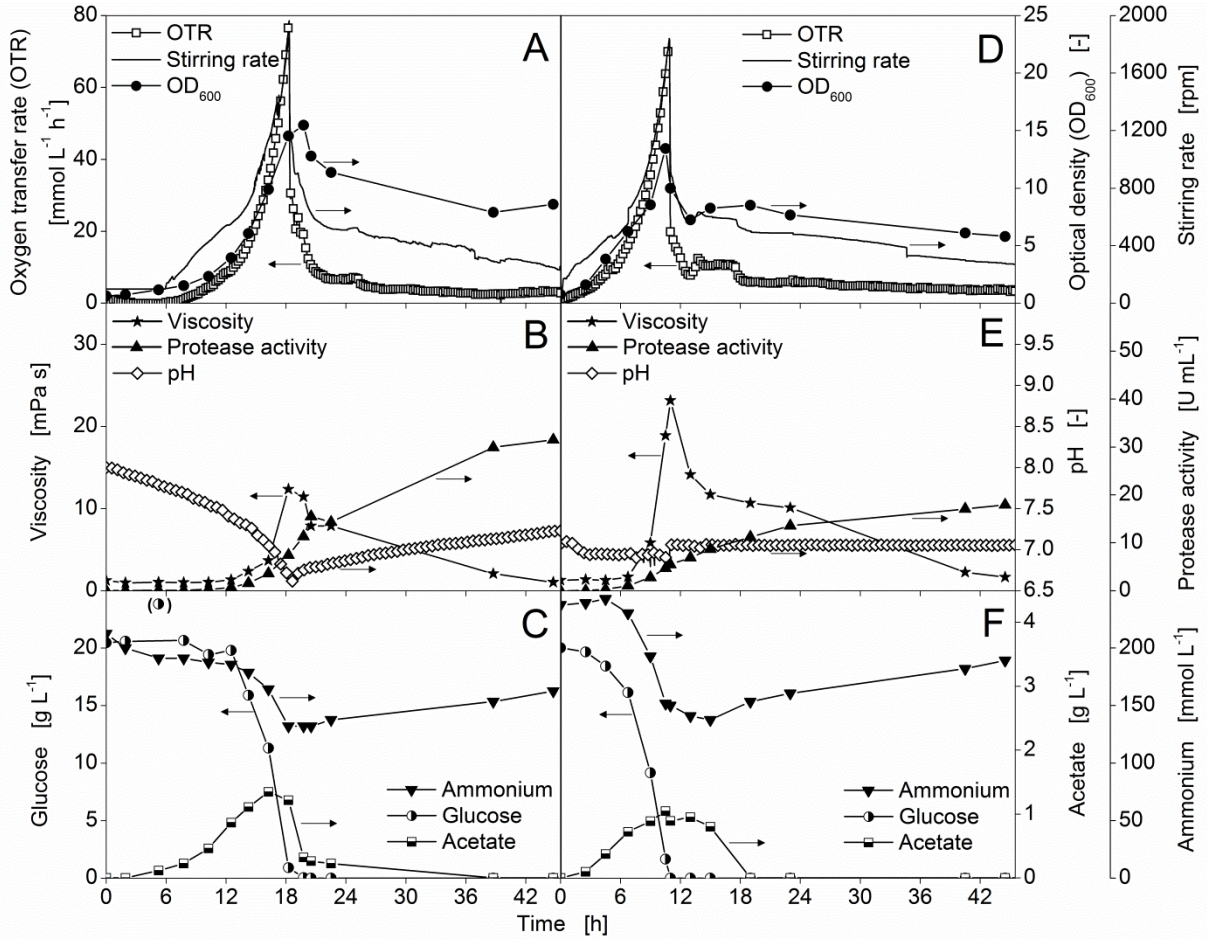

Figure 3-4 Impact of pH control on viscosity build-up due to poly(γ-glutamic acid) formation during fermentation in a mineral medium containing ammonium as nitrogen source

Comparison of *B. licheniformis* cultivations in 3 L stirred tank bioreactors. Cultivations were performed using either buffer **(A-C)** or pH titration **(D-F)**. For cultivations with buffer, 0.2 M MOPS buffer was used, and the initial pH was set to 8. In case of titration, the pH was controlled at pH 7 with 2 M NaOH and 2 M HCl. The DOT was maintained above 30 % air saturation by controlling the stirring rate. Oxygen transfer rate (OTR), optical density (OD_{600}), and stirring rate are shown in **(A)** and **(D)**. Viscosity at a shear rate of 300 s^{-1}, protease activity, and pH are presented in **(B)** and **(E)**. Glucose, acetate, and ammonium concentrations are shown in **(C)** and **(F)**. In the fermentation with buffer, a maximum acetoin concentration of 0.35 g L^{-1} was detected after 44.75 h; 2,3-butanediol was not detected in any of the samples **(A-C)**. In the pH controlled fermentation, maximum acetoin and 2,3-butanediol concentrations of 0.23 g L^{-1} and 0.17 g L^{-1} were detected after 11 h and 10.5 h, respectively **(D-F)**. Initial values: OD_{600} = 0.4, 20 g L^{-1} glucose, 15 g L^{-1} $(NH_4)_2SO_4$. Cultivation conditions: T = 37 °C, filling volume 1.7 L.

The ammonium concentration reached a minimum of 132 mmol L^{-1} after 18 h which corresponds with the pH minimum of 7.1 at the same time (Fig. 3-4B and C). From there, the ammonium concentration increased again to a final value of 162 mmol L^{-1} due to the

consumption of glutamic acid released from the degradation of γ-PGA and maybe partly also due to cell lysis, indicated by a decreasing OD_{600}. The final protease activity was 31 U mL^{-1} (Fig. 3-4B) and the maximum protease formation rate was 204 U h^{-1} g^{-1} CDW after 19 h of cultivation.

In the fermentation with titration, the pH value was controlled at pH 7.0 (Fig. 3-4E). The lag phase was shorter than for the fermentation with buffer and was similar to the reference shake flask cultivation. Progression curves of OTR, OD_{600}, glucose, and acetate are also comparable to the reference process. For this cultivation, a viscosity of maximal 23 mPa s was observed. No precipitation of medium components was observed, likely because the pH value was neutral (alkaline pH values favor the formation of precipitates). The ammonium concentration, starting at 238 mmol L^{-1}, reached a minimum of 138 mmol L^{-1} after 15 h and increased again to a final concentration of 189 mmol L^{-1} (Fig. 3-4F). In this fermentation, a protease activity of only 18 U mL^{-1} was reached, which is roughly 50 % compared to the respective reference cultivation in shake flasks (Fig. 3-1A-C). It is not yet clear why the protease activity was relatively low in this experiment. The maximum protease formation rate was also rather low with 146 U h^{-1} g^{-1} CDW at 13.8 h of cultivation.

For nitrate as nitrogen source, the same kind of scale-up from 250 mL shake flasks to a 3 L stirred tank bioreactor was conducted. Figures 3-5A-C display the result of a stirred tank bioreactor fermentation with nitrate as nitrogen source and titration for pH control. Identical to the fermentation with ammonium and titration, the pH value was controlled at pH 7.0. Analogous to the fermentation depicted in Figs. 3-4A-C, a fermentation with buffer for pH control was conducted as well (data not shown) and showed the general transferability of results from the shake flask to the stirred tank bioreactor. In the fermentation with titration for pH control, no viscosity build-up, i.e. no viscosity elevating by-product formation of γ-PGA, was observed (Fig. 3-5B). The nitrate concentration decreased from 229 mmol L^{-1} to a minimum of 167 mmol L^{-1} after 15 h and then remained constant until the end of the fermentation (Fig. 3-5C). The final protease activity was 36 U mL^{-1}, which corresponds to 95 % of the reference shake flask cultivation with ammonium, buffered with 0.2 M MOPS. A maximum protease formation rate of 318 U h^{-1} g^{-1} CDW at 11.6 h of cultivation was obtained.

In the experiments shown, two pH control systems – buffer and titration – were compared regarding their influence on the undesired γ-PGA formation. The two pH control systems corresponded to a changing or a constant pH progression curve. From the obtained results, it can be concluded that independent of the applied pH control system, the viscosity elevating by-product γ-PGA is formed when ammonium is applied as the nitrogen source. The results confirm the hypothesis that nitrate prevents the formation of the undesired by-product γ-PGA, while ammonium promotes it.

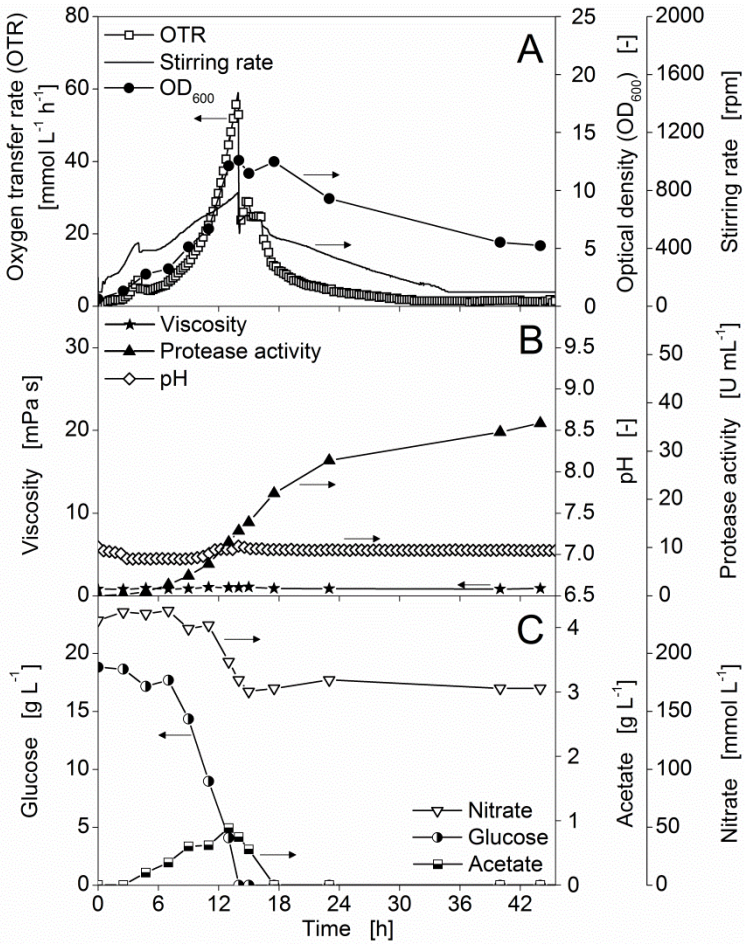

Figure 3-5 Impact of pH control on viscosity build-up due to poly(γ-glutamic acid) formation during fermentation in a mineral medium containing nitrate as nitrogen source

Cultivation of *B. licheniformis* in a 3 L stirred tank bioreactor with titration for pH control. The pH was controlled at pH 7 with 2 M NaOH and 2 M HCl. The DOT was maintained above 30 % air saturation by controlling the stirring rate. Oxygen transfer rate (OTR), optical density (OD_{600}), and stirring rate are shown in **(A)**. Viscosity at a shear rate of 300 s^{-1}, protease activity, and pH are presented in **(B)**. Glucose, acetate, and nitrate concentrations are shown in **(C)**. A maximum acetoin concentration of 0.16 g L^{-1} was detected after 40 h; 2,3-butanediol was not detected in any of the samples. Initial values: OD_{600} = 0.4, 20 g L^{-1} glucose, 22.95 g L^{-1} KNO_3. Cultivation conditions: T = 37 °C; filling volume 1.7 L.

3.3 Influence of oxygen limitation on viscosity build-up in shake flask fermentations with nitrate as nitrogen source

If the substitution of ammonium by nitrate is actually preventing the formation of the viscosity elevating by-product γ-PGA, then the mechanism might be as follows: as shown in Fig. 3-3, the use of nitrate as nitrogen source is far more energy consuming than the use of ammonium. The cells will, therefore, only take up nitrate and reduce it to ammonium in necessary amounts. Thus, no excess ammonium is available for excess glutamate formation which can be directed to γ-PGA production. However, under conditions of an oxygen limitation – reduced oxygen supply, not anaerobic conditions – reduced cofactors (NAD(P)H) might not be re-oxidized by the respiratory chain with the oxygen molecules available. For this reason, the concentration of reduced cofactors in their reduced form is usually higher in cells suffering from oxygen limitation compared to cells under oxygen unlimited conditions (Asali et al., 1992; Harrison and Chance, 1970). However, if nitrate is provided as nitrogen source for cells grown under oxygen limited conditions, those cells might use nitrate as an electron acceptor for the reduced cofactors which can otherwise not be oxidized due to a lack of oxygen (or other electron and proton acceptors). Therefore, more ammonium would be available for these cells than for cells grown under oxygen unlimited conditions. Consequently, this ammonium could be used for γ-PGA formation. Li et al. (Li et al., 2014) found just recently that the addition of nitrate to a mineral medium containing also ammonium enhanced γ-PGA production of a *B. licheniformis* strain more than 2-fold compared to the medium without nitrate addition. The cultivations were carried out under oxygen limited conditions. The improved production was attributed to the reduction of nitrate which was suggested to act as an additional electron acceptor besides oxygen and to provide additional ammonium for γ-PGA production.

To test the above mentioned hypothesis, a shake flask experiment was conducted with nitrate as the sole nitrogen source. An oxygen limitation was imposed by reducing the shaking frequency from 350 to 200 rpm and increasing the filling volume from 10 to 25 mL compared to the reference cultivations in Fig. 3-1. The results of this experiment are depicted in Fig. 3-6A-C. Due to higher filling volume and lower shaking frequency, the maximum OTR reached was only 19 mmol L^{-1} h^{-1} (Fig. 3-6A).

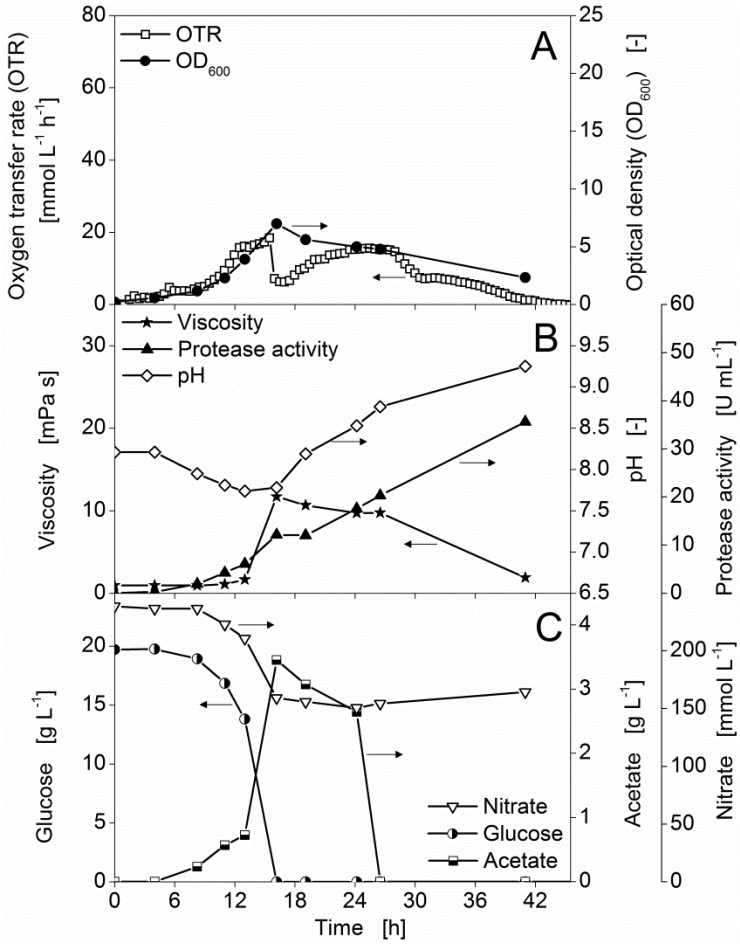

Figure 3-6 Impact of oxygen limitation on viscosity build-up due to poly(γ-glutamic acid) formation during fermentation in a mineral medium containing nitrate as nitrogen source

Cultivation of *B. licheniformis* in shake flasks with medium containing 0.2 M MOPS buffer. The filling volume was elevated and the shaking frequency reduced to impose oxygen limitation. Oxygen transfer rate (OTR) and optical density (OD_{600}) are shown in **(A)**. Viscosity at a shear rate of 300 s^{-1}, protease activity, and pH are presented in **(B)**. Glucose, acetate, and nitrate concentrations are shown in **(C)**. Neither acetoin nor 2,3-butanediol were detected in any of the samples. Initial values: OD_{600} = 0.4, 20 g L^{-1} glucose, 22.95 g L^{-1} KNO_3. Cultivation conditions: T = 37 °C, 250 mL shake flasks, filling volume 25 mL, shaking frequency 200 rpm, shaking diameter 50 mm.

The beginning of the oxygen limited phase is indicated by the plateau of the OTR curve starting at approximately 12 h of cultivation. The sharp decrease after 16 h is due to the depletion of glucose (Fig. 3-6C). Because of the oxygen limitation, more acetate was formed than in all the other presented experiments which were conducted under oxygen unlimited conditions. The maximum acetate concentration observed was 3.5 g L^{-1}. Due to acetate production, the pH decreased during the fermentation to a minimum of 7.7 (Fig. 3-6B). The nitrate concentration, starting at 239 mmol L^{-1}, decreased to 159 mmol L^{-1} after 16 h and

remained constant until the end of the cultivation (Fig. 3-6C). Most importantly, a maximum viscosity build-up of 12 mPa s after 16 h was observed, indicating the formation of the by-product γ-PGA (Fig. 3-6B). This maximum coincides with the depletion of glucose and the maximum acetate concentration. The OD_{600} increased to a maximum of 7.0 until the end of the first growth phase at 16 h. Subsequently, a distinctive second phase of respiration activity is visible, depicted by a second OTR plateau. During this phase, acetate and γ-PGA produced during the first growth phase are consumed. The consumption of both by-products led to an increase of the pH value up to pH 9.3. Due to the consumption of γ-PGA, the viscosity decreased. At the end of the fermentation, the initial viscosity of ~1-2 mPa s was restored. The OD_{600} also decreased slowly to a final value of 2.3, probably due to morphological changes of the cells. A final protease activity of 36 U mL^{-1} was achieved (Fig. 3-6B). The maximum protease formation rate was 386 U h^{-1} g^{-1} CDW after 9.6 h of cultivation.

The maximum protease formation rates were generally lower in the experiments with ammonium as nitrogen source (146-262 U h^{-1} g^{-1} CDW) compared to the experiments with nitrate (318-472 U h^{-1} g^{-1} CDW). In the ammonium experiments, the protease activity increased relatively evenly during the whole fermentation. In some experiments, the largest portion of protease was even produced in the second part of the cultivation during the longer growth phase on γ-PGA, and not in the first, shorter, growth phase on glucose (compare Fig. 3-1B). In the nitrate experiments, half or even more of the protease was produced in the first growth phase on glucose (compare Fig. 3-1E). The exemption is the experiment with nitrate under oxygen limited conditions, where more than half of the protease was produced in the second growth phase on γ-PGA and acetate.

The increase of viscosity in the experiment depicted in Fig. 3-6B strongly supports the hypothesis that under oxygen limiting conditions more nitrate is reduced to ammonium, which is then available for γ-PGA formation. An influence of oxygen limitation on γ-PGA production was also recently demonstrated by Wilming et al. (2013) in a medium with ammonium as nitrogen source. Catabolite controlled overflow was identified as one of the key triggers for γ-PGA formation which was further potentiated if an oxygen limitation was imposed. The results of this work clearly fortify the findings of Wilming et al. (2013), that the combination of high glucose and high ammonium concentrations triggers the overflow metabolism of *B. licheniformis* leading to the production of γ-PGA. Therefore, strictly limiting

either glucose or ammonium by applying a fed-batch mode for one or even both nutrients should prevent the formation of undesired γ-PGA in fermentations. The stirred tank bioreactor experiments in this study, however, were explicitly conducted in batch mode to adjust equal conditions as in the shake flask cultivations and uncouple the influence of nitrogen source and pH value from each other at the same time. Under these conditions, nitrate could clearly be identified as the sole factor which prevents γ-PGA formation under oxygen unlimited conditions. Additionally, nitrate as a means to limit the availability of ammonium to the cellular metabolism can very easily be applied in shake flask cultivations compared to an ammonium fed-batch. Since screening experiments and the first steps of process optimization are usually performed in batch mode in small scale cultivation systems (Klöckner and Büchs, 2012), the substitution of ammonium by nitrate represents a simple tool to prevent undesired γ-PGA formation. Furthermore, this method is scalable to stirred tank bioreactors. Large scale protease production processes, as many other production processes, are generally performed in fed-batch mode. If the fed-batch fermentation is strictly carbon or ammonium limited, no γ-PGA formation should occur. However, some practical protease production processes are performed with glucose feeding, but are not strictly carbon limited and sometimes cope with the problem of undesirable γ-PGA formation (personal communication). In that case, the application of nitrate instead of ammonium or ammonium containing complex compounds as nitrogen source should prevent undesired γ-PGA production.

3.4 Summary

The *B. licheniformis* strain A1 is an industrial protease producer that is also able to secrete γ-PGA as an undesired by-product under standard fermentation conditions. The applied medium contained ammonium as the sole nitrogen source and glucose as the carbon source, from which *B. licheniformis* apparently synthesizes glutamate as an overflow metabolite. In the presence of this excess of glutamate, γ-PGA is formed. If, instead of ammonium, nitrate was added as the sole nitrogen source, no γ-PGA formation was observed. Thus, the substitution by nitrate successfully prevented the undesired γ-PGA formation. Before assimilation, nitrate must be reduced to ammonium. Therefore, it has distinctively higher energy costs than ammonium and will only be taken up and reduced in the amounts required for cell growth and maintenance. With nitrate, no excess glutamate is formed and, thus, no γ-PGA is produced.

The application of ammonium or nitrate as nitrogen sources resulted in different pH progression curves. Therefore, the influence of the pH value on the undesired γ-PGA was investigated in order to distinguish between the impact of the nitrogen source and the impact of the pH value. The experiments conducted in stirred tank bioreactors with either a buffer system or titration for pH control revealed that the pH value has no influence on preventing or promoting the undesired γ-PGA formation.

All of these experiments were performed under oxygen unlimited conditions. But when *B. licheniformis* was cultivated under oxygen limited conditions and with nitrate as nitrogen source, γ-PGA formation was observed. Under oxygen limited conditions, reduced cofactors like NAD(P)H accumulate due to a lack of oxygen that acts as the usual electron acceptor necessary to regenerate the reduced cofactors. Other molecules such as nitrate can also act as terminal electron acceptors. The accumulated reducing agents can be oxidized by reducing nitrate to ammonium, which the cells can then use in the biosynthesis of glutamate and γ-PGA. Under oxygen limited conditions, therefore, γ-PGA could be formed despite the fact that nitrate was applied as nitrogen source.

The experiments showed that undesired γ-PGA formation of a protease producing *B. licheniformis* strain can be prevented by simply exchanging the nitrogen source in the applied mineral production medium. This change in medium composition is applicable for shake flask and stirred tank bioreactor fermentations, respectively, since the typically used pH control systems with buffer or titration did not have an influence on the undesired γ-PGA formation.

Chapter 4

Investigation of different factors influencing the poly(γ-glutamic acid) production by *B. licheniformis* ATCC 9945

The strain *B. licheniformis* ATCC 9945 is commonly known for its γ-PGA production and is publicly available from a strain collection. Several publications have dealt already with different aspects of this strain as a γ-PGA producer (Birrer et al., 1994; Cromwick et al., 1996; Cromwick and Gross, 1995; Leonard et al., 1958; Thorne et al., 1954; Troy, 1973a, b). However, for shake flask cultivations, no online measurement data are available in the literature so far. In this work, the production process of γ-PGA formation of *B. licheniformis* ATCC 9945 in shake flask cultivations was thoroughly investigated using a RAMOS device for online measurement of the OTR and intensive offline sampling for determination of carbon source consumption, γ-PGA production (via viscosity measurements), formation of by-products acetate, acetoin, and 2,3-butanediol, as well as biomass formation. Moreover, the pH value and the consumption of ammonium and phosphate were monitored. From these results, the influence of different medium compounds on γ-PGA production was suggested and further investigated. In Chapter 4.1, the results of a cultivation under so-called standard conditions are presented. In Chapter 4.2, the impact of a higher phosphate concentration is shown and in Chapter 4.3, the influence of a higher citric acid concentration. Chapter 4.4 presents the investigation of a higher citric acid concentration combined with either a higher phosphate or a higher ammonium concentration. Chapter 4.5 shows the influence of different filling volumes.

Chapter 4.6 closes with a summary. The presented experiments were partly conducted by Anna-Lena Altenhoff and Hannah Braß (research internships).

4.1 Reference cultivation under standard conditions

In this chapter, the results of a cultivation under "standard conditions" are presented. These cultivation conditions were used as a reference for the different medium compositions and cultivations conditions which were investigated afterwards. Standard conditions were defined as cultivations in shake flasks of *B. licheniformis* ATCC 9945 in mineral medium E (Leonard et al., 1958) with shaking conditions similar or identical to those often found published for production of γ-PGA (Birrer et al., 1994; Cromwick and Gross, 1995; Shih et al., 2002). These shaking conditions include 500 mL shake flasks (without baffles), a filling volume of 100 mL, and a shaking frequency of 250 rpm. The applied shaking diameter is unfortunately not mentioned in any of the publications. For this work, a shaking diameter of 50 mm was chosen, since smaller shaking diameters favor the out-of-phase phenomenon, especially for viscous cultivation systems (Büchs et al., 2001). Also, it was known from oral communication, that Margaritis et al., who published several studies about γ-PGA production with *B. licheniformis* (Buescher and Margaritis, 2007; Manocha and Margaritis, 2010; Richard and Margaritis, 2003a, b) used a shaking diameter of 50 mm. For cultivations, the mineral medium was directly inoculated from glycerol stocks without any pre-culture which is in accordance with the respective information in the literature mentioned above.

Figure 4-1 shows the results of the cultivation of *B. licheniformis* ATCC 9945 under the standard conditions described above. At each sampling time point, three shake flasks were withdrawn from the shaker for offline analysis (and not placed back). The depicted values are the mean of the three shake flasks. Figure 4-1A shows the OTR curves of five in parallel cultivated RAMOS shake flasks. After a short lag phase, the OTR increased exponentially up to 8.5 mmol L^{-1} h^{-1} after 9.5 h. Then, the OTR deviated to a further non-exponential increase up to 11 mmol L^{-1} h^{-1} which was reached after about 22 h. From this time point until the end of the cultivation, the OTR showed an almost constant plateau, indicating a state of oxygen limitation. Despite the filling of the RAMOS shake flasks from the same master mix, the OTR curves are only running in parallel until 130 h.

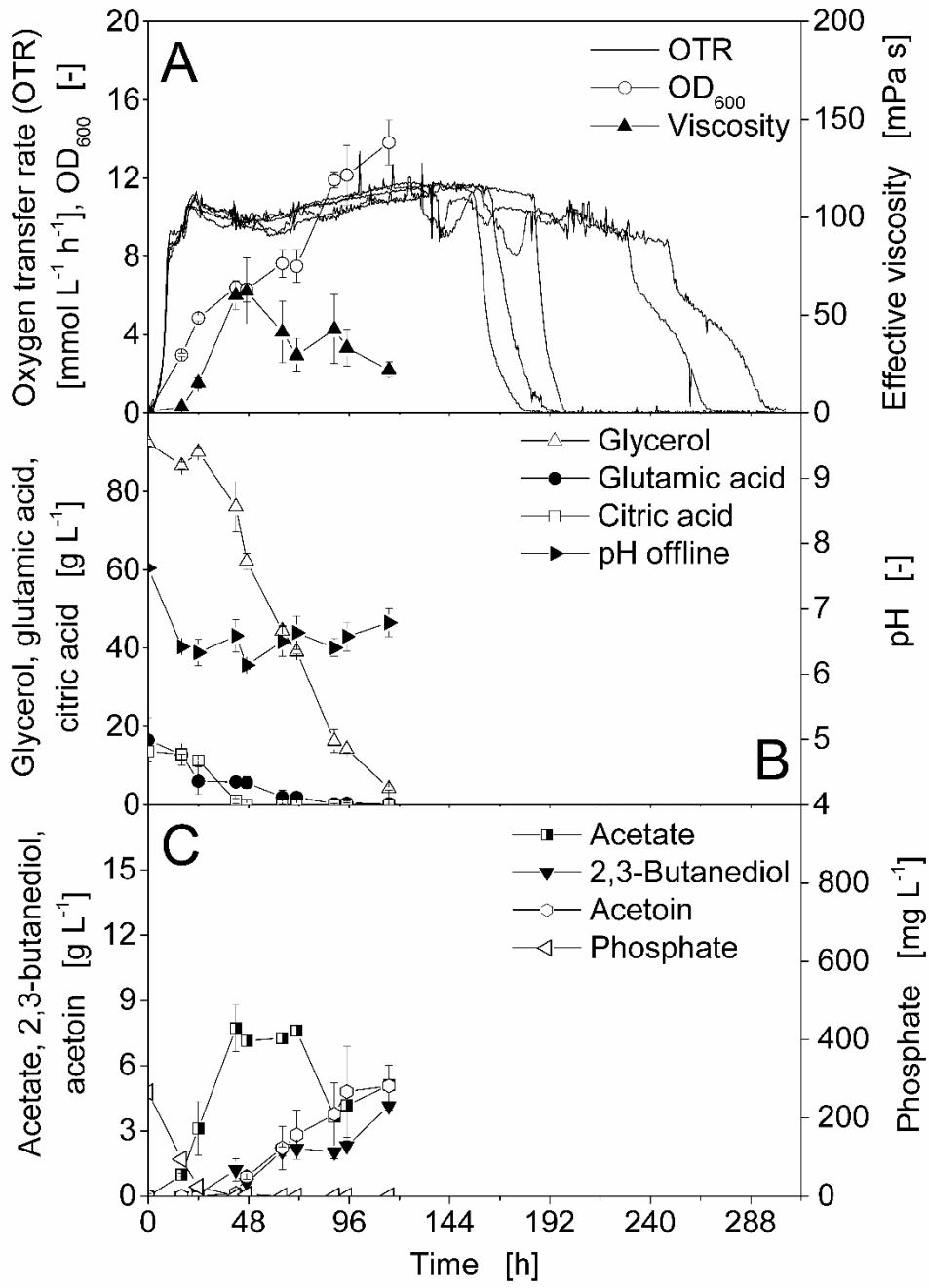

Figure 4-1 Growth and γ-PGA production of *B. licheniformis* ATCC 9945 in standard mineral medium E

Oxygen transfer rates (OTR) of five parallel flasks, optical density (OD_{600}), and effective viscosity are shown in **(A)**. Carbon source concentrations of glycerol, glutamic acid, and citric acid, as well as the pH value are shown in **(B)**. Phosphate concentration and overflow metabolite concentrations of acetate, acetoin, and 2,3-butanediol are presented in **(C)**. Initial values: pH = 7.6, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from glycerol stock (0.4 % v/v).

For some flasks, the OTR curves show drops to about 8 mmol L^{-1} h^{-1} for several hours which probably indicates a shift between the consumption and/or production of different carbon sources and by-products, respectively. Between 155 to 185 h, the OTR of three of the five shake flasks started to decrease, resulting in the end of respiration activity for these cultures. The decrease to 0 mmol L^{-1} h^{-1} is not sudden, as it is usually observed for the depletion of the carbon source(s), but took several hours. This rather indicates that respiration activity ceased due to some kind of inhibition of the cells or a lack of another nutrient necessary for metabolic activity. At the same time, the other two flasks show the aforementioned drops in their OTR curves but continued in their respiration activity until 230 and 250 h, respectively. The OTR curves then also decreased slowly to 0 mmol L^{-1} h^{-1}.

A deviation of the OTR curves of parallel cultivated shake flasks was observed in all experiments conducted under standard conditions. Only the time point of the diverging varied, occurring at the earliest after about 60 h of cultivation. Figure 7-1 in the appendix depicts the OTR curves of all cultivations performed under standards conditions in one diagram. The phenomenon of the deviating OTRs could easily be observed with the RAMOS device but would have been more difficult or even impossible to identify if only offline sampling was performed. The pH value proved to be the only reliable offline indicator to determine whether a culture had terminated the respiration activity during cultivation or only at the end. It was observed that cultures in RAMOS shake flasks which showed an early decrease in OTR had a pH value of 5.5 or lower. Shake flasks with OTR decreases after 216 h exhibited pH values generally higher than pH 9. After this correlation had been identified, it was also possible to decide with a high probability whether the cultures of the shake flasks used for offline analysis belonged to the first (low final pH = early termination of respiration activity) or second group (high final pH = late termination). In the experiment depicted in Fig. 4-1, the final pH value of the three flasks with an earlier decrease of the OTR was approximately 5.3, while the final pH value of the other two flasks was above 9.2.

The OD_{600} increased exponentially during the first 24 h and after that linearly, as the cultures grew under oxygen limited conditions. The highest OD_{600} of 13.8 was measured for the last sampling time point after 115 h (Fig. 4-1A).

γ-PGA production was measured indirectly by determining offline the effective viscosity. The viscosity increased only slightly during the first 16 h of cultivation, but more strongly until approximately 48 h when the sharp maximum of 62 mPa s was reached (Fig. 4-1A). After that, viscosity directly decreased again. After 115 h, at the end of sampling, a viscosity of 24 mPa s was measured. Thus, γ-PGA is first produced and apparently degraded later on and possibly also consumed. Two γ-PGA degrading enzymes from γ-PGA producing *Bacillus subtilis* strains have been identified: one is an endopeptidase-like depolymerase which cleaves the polymer into smaller oligomers (Ashiuchi et al., 2003b). These oligomers can be further cleaved by an exopeptidase into single glutamate monomers (Abe et al., 1997). A rapid decrease of molecular weight of the produced γ-PGA during cultivation was observed in other studies. There, the highest molecular weight was measured after approximately 24 h (Cromwick and Gross, 1995; Gross and Ko, 1998). A strong decrease of molecular weight of γ-PGA would result in a significant decrease of viscosity, as it was observed in this work. As a reason for the decrease of the molecular weight it was suggested that *B. licheniformis* ATCC 9945 produces γ-PGA depolymerases during cultivation (Cromwick and Gross, 1995; Gross and Ko, 1998). It can, therefore, be concluded, that the decrease of the effective viscosity is most probably due to the formation of γ-PGA degrading enzymes.

The applied mineral medium E contains three carbon sources: glycerol, glutamic acid, and citric acid. All three were consumed in parallel (Fig. 4-1B). The first carbon source which was depleted was citric acid after about 48 h. Glutamic acid was depleted next after 90 h. At 115 h, the glycerol concentration was below 5 g L^{-1}.

The starting pH value in this experiment was 7.6 (Fig. 4-1B). The initial pH value of medium E according to the reference (Leonard et al., 1958) is 7.4. When preparing the medium, the pH value was set to 7.4 before autoclaving, checked afterwards, and if necessary, re-set to 7.4. However, when the pH was already close to 7.4 it was not corrected. Moreover, adding the glycerol stocks to the medium often changed the pH value slightly. Therefore, the starting pH values of the different experiments were not always exactly at 7.4 and are given in the respective figure captions. During the first 24 h, the pH value decreased sharply to 6.3. The minimum pH value of 6.1 was measured at 48 h. Thereafter, the pH value increased again up to 6.8 after 115 h.

The phosphate and also the ammonium concentration were measured to determine whether a limitation of one of these macro nutrients occurs. Medium E contains about 2400 mg L^{-1} ammonium and 270 mg L^{-1} phosphate. The ammonium concentration decreased significantly during the first 48 h when γ-PGA was produced, but the concentration never fell below 900 mg L^{-1} (data in appendix, Fig. 7-3). Therefore, no ammonium limitation occurs for cultivations under standard conditions. Figure 4-1C depicts the phosphate consumption and the formation of overflow metabolites during cultivation. Due to biomass formation, the phosphate concentration also decreased strongly at the beginning of the cultivation. At 42 h, already a phosphate concentration below 1 mg L^{-1} was determined. The phosphate concentration remained limited until the end of the sampling at 115 h.

Besides γ-PGA, also certain overflow metabolites were formed as by-products. Typical for *Bacillus*, these were acetate, acetoin, and 2,3-butanediol (Sonenshein, 2007). Acetate is produced first, followed by 2,3-butanediol and acetoin (Fig. 4-1C). At 42 h, 7.7 g L^{-1} acetate were measured. The acetate concentration remained at this level for 30 h and decreased slightly afterwards. At 115 h, 5.1 g L^{-1} were still found in the fermentation broth. For acetoin and 2,3-butanediol, the highest concentrations of 5.1 and 4.2 g L^{-1} were measured at 115 h. Further experiments (see also Chapter 5) showed that all three by-products usually were not only produced during cultivation but also consumed. However, at the end of the cultivations one or more by-products were often still detectable in the fermentation broth although respiration activity of the cultures had already ceased.

The results of the reference cultivation depicted in Fig. 4-1 clearly show that at a certain time point during cultivation, the single cultures do not run in parallel anymore but instead diverge in their respiration and metabolic activity, respectively. The early termination of the respiration activity coincided with a very low pH value (<5.5). This phenomenon was also observed for other cultivations under standard conditions and for cultivations with different medium compositions, of which the results are depicted and discussed in following chapters. Nevertheless, since the phenomenon is first described in the current chapter, it will be discussed in more detail in the subsequent paragraphs.

It is not clear so far, why some of the cultures – which all have their origin in the same mastermix – fail to cope with the low pH value and others are able to recover and cause an

increase of pH value. The divergence of the supposedly identical cultures could be due to various cell types or subpopulations being present in the initial culture. Minor differences during the cultivation might subsequently result in the major divergence of respiration activities. An event, which would result in different subpopulations, is the induction of spore formation. The applied *B. licheniformis* strain ATCC 9945 is a wildtype strain and harbors active sporulation genes. For the wildtype *B. subtilis* strain Marburg, it was observed that in exponentially growing cultures, the bacterial population consisted of three types: growing cells, non-growing cells committed to sporulate, and spores (Schaeffer et al., 1965). In this work, spore formation was also observed during cultivation by taking microscopic pictures of the cultures. Often, spores were observed for the first time after approximately 40 h, but sometimes already after 24 h. The number of spores increased steadily during cultivation, from very few spores inside the cells, to more frequent spores inside the cells and also free spores. However, the number of cells usually exceeded the number of spores, according to an approximate estimation of the microscopic pictures. But sometimes, a large number of spores was observed towards the end of the cultivation in cultures with still active respiration and, therefore, high pH values (pH > 8). Cultures with terminated respiration activity exhibited less spores and also less cells in general than active cultures. Also, samples from those cultures contained more cell debris or cells which appeared damaged due to their nonuniform coloring.

Chemostat experiments with the *B. subtilis* strain Marburg showed that sporulation occurred with high frequency under glucose or nitrogen limited conditions. Spore formation also increased with decreasing dilution rate and, therefore, decreasing growth rate. Interestingly, a limitation of phosphate, citric acid, or Mg^{2+} did not result in increased sporulation (Dawes and Mandelstam, 1970). However, sometimes phosphorous starvation, besides limitation of carbon or nitrogen source, was also named as a trigger for spore formation in *B. subtilis* (Piggot and Coote, 1976; Piggot and Hilbert, 2004). Therefore, the depletion of citric acid is likely not responsible for the formation of the observed spores and can probably be excluded as the reason for the divergence of the cultures. The depletion of phosphate could possibly play role. However, in an experiment with a non-limiting phosphate concentration (Chapter 4.2), also a divergence of the respiration activities was observed. Under standard conditions, neither a carbon nor a nitrogen limitation could be stated. But the increase in the number of spores during cultivation could be due to a decreasing growth rate. Hence, the sporulation process should seriously be considered as a reason for the divergence in respiration activities.

For *B. licheniformis*, it was observed that different cell morphologies existed in the same culture on agar plates. The cells differed in their size and motility: short, motile cells with flagella and chains of cells which were non-motile (Bisset and Street, 1973). For the cultures in this work, also different cell types were observed by microscopy. At the beginning of cultivations, predominantly short cells and a few longer cells were present. Subsequently, less short and more long cells appeared. Short cells often were present as single cells, while the longer cells existed mainly as doubles or chains of cells. Bisset and Street (1973) also noticed that spores were produced more frequently in the short cells and only rarely in the filamentous-like cells. Considering these observations, it is not clear what is cause and what is effect. Do different phenotypes of cells – short or long – reflect different transcriptomes and, subsequently, result in different sporulation frequencies? Or does a difference in the transcriptome of the sporulation machinery lead to different cell phenotypes, namely short and long cells? Therefore, different cell types and spore formation could be closely related phenomena, where one depends on the other.

In this work, both aspects – sporulation and different cell types – were observed for the cultures of *B. licheniformis* ATCC 9945. To answer the question whether one or both of these aspects are responsible for the divergence of the cultures, it is useful to take a look at a non-sporulating *B. licheniformis* strain. In the strain A1, which was introduced in Chapter 3, one for the sporulation process important gene, *spo0A*, was deleted, and sporulation thereby inactivated. Cultivations of A1 under standard conditions showed much more reproducible curves of respiration activity (Chapter 5.3). The final pH values of the cultures were usually above 9; there were only very few exemptions of cultures with low final pH values. The cultures of A1 also showed different cell types during cultivation. All these observations clearly suggest that spore formation, and the gene regulation associated with the whole process of spore formation, is responsible for or at least participates in the divergence of the cultures, resulting ultimately in the early termination of respiration activity of some of them.

For cultivations of ATCC 9945 under standard conditions, the maximum effective viscosity was obtained at 48 h and, hence, earlier than the earliest deviation of the cultures which was observed after 60 h. Thus, it can be concluded that the γ-PGA production phase is comparable for parallel running cultures and the diverging does not interfere with the production process. The decrease in viscosity after the maximum is reached shows that an unknown factor

apparently triggers the enzymatic degradation of the produced γ-PGA. The results also showed that the maximum viscosity coincided with the depletion of one of the carbon sources – citric acid. Moreover, phosphate is already limiting at that time. In following experiments, the influence of higher phosphate and citric acid concentrations on γ-PGA production was investigated to determine whether the limitation of one or both of these nutrients is important for the production process and acts as a trigger for the enzymatic degradation process.

4.2 Influence of additional phosphate

During the reference cultivation, it became apparent that a phosphate limitation occurred which coincided with the maximum viscosity (see Chapter 4.1). In the experiment presented in this chapter, the phosphate concentration in the medium was tripled to investigate the influence of unlimited phosphate conditions on the γ-PGA production process. Figure 4-2 shows the comparison of a cultivation under standard conditions and a cultivation with tripled phosphate concentration. Except for the different amount of phosphate, all cultivation conditions were identical. The two approaches were run in parallel. For each approach, four RAMOS shake flasks were used. Therefore, four OTR curves are depicted, respectively. For each sampling point, two shake flasks were withdrawn from the shaker and not placed back. The shown offline values are the mean of the two flasks.

For the standard conditions, all OTR curves increased exponentially after a short lag phase and reached 11 mmol L^{-1} h^{-1} after 11 h (Fig. 4-2A). The OTR continued on this level, showing the state of oxygen limitation already described in the chapter before. In this experiment, all four cultures in the RAMOS shake flasks showed a very early decrease in respiration activity, which already started after about 60 h, and reached 0 mmol L^{-1} h^{-1} after 96 h. In contrast, the OTR curves of the cultures in medium E with tripled phosphate showed an identical progression at the beginning, but only one of the OTR curves decreased early (after about 60 h), while the other three curves continued on the level of oxygen limitation until 160 h (Fig. 4-2D). In between, some of the OTR curves showed drops down to lower OTR values, as was already observed for the reference cultivation in Chapter 4.1. Although on a first glance, it might seem that more phosphate is beneficial for a prolonged respiration activity of the cultures, Chapter 4.1 revealed that in other experiments conducted under standard conditions

the cultures showed respiration activity as long as the tripled phosphate cultures and even longer (see Fig. 7-1, appendix).

For the standard conditions, the OD_{600} increased linearly throughout the cultivation and the maximum of 8.8 was obtained at 96 h (Fig. 4-2A). The OD_{600} of the tripled phosphate approach had its maximum of 7.6 after 66 h. At the end of the sampling time frame after 116 h, an OD_{600} of 3.8 was determined (Fig. 4-2D).

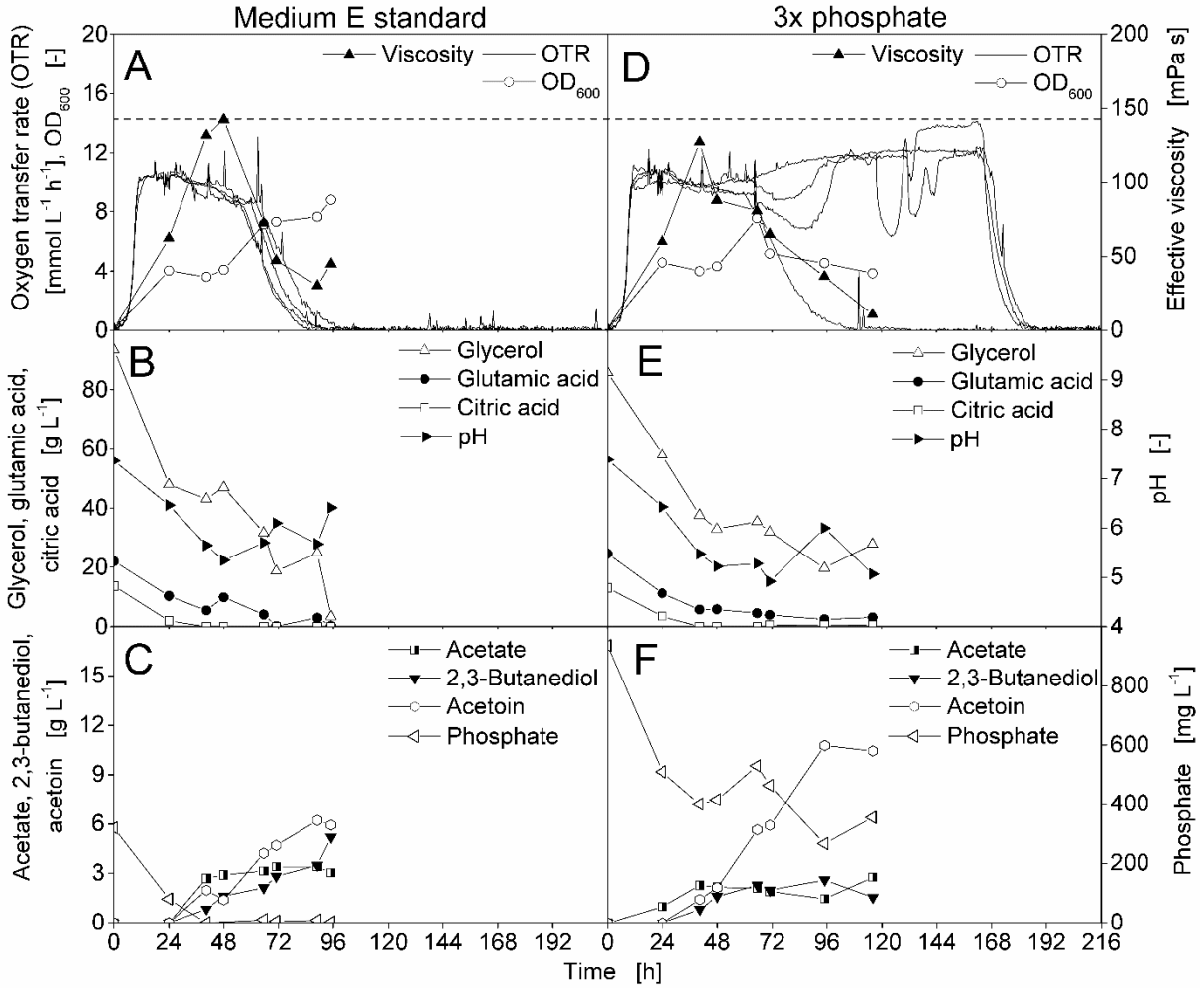

Figure 4-2 Impact of additional phosphate on γ-PGA production of *B. licheniformis* ATCC 9945

Oxygen transfer rates (OTR), optical density (OD_{600}), and effective viscosity are shown in **(A)** and **(D)**. Carbon source concentrations of glycerol, glutamic acid, and citric acid, as well as the pH value are shown in **(B)** and **(E)**. Phosphate concentration and overflow metabolite concentrations of acetate, acetoin, and 2,3-butanediol are presented in **(C)** and **(F)**. Initial values: pH = 7.4, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 **(A-C)** or 1.5 **(D-F)** g L^{-1} K_2HPO_4. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from glycerol stock (0.4 % v/v).

The maximum effective viscosity of the standard conditions was 142 mPa s, measured after 48 h. Then, the viscosity decreased again significantly (Fig. 4-2A). For the tripled phosphate cultures, the maximum viscosity was only 127 mPa s, determined after 41 h. The viscosity thereafter also decreased rapidly and reached 11 mPa s after 116 h (Fig. 4-2D). The increased phosphate concentration therefore had no positive effect on γ-PGA formation; maybe even a slightly negative one. Possibly, the phosphate limitation is necessary or at least beneficial for the production process.

The carbon source consumption patterns for both approaches are similar, however, there are also small differences (Figs. 4-2B and E). In both cases, all three carbon sources are consumed in parallel and citric acid is depleted after 42 h. For the standard conditions, glutamic acid is depleted after 96 h and the glycerol concentration is below 5 g L^{-1}. For the tripled phosphate conditions, 2.5 g L^{-1} glutamic acid and 19.8 g L^{-1} glycerol were still measured at that time. Therefore, carbon source consumption was slower for the cultures with increased phosphate concentration. The starting pH value for both conditions was 7.4 (Figs. 4-2B and E). For the standard conditions, the minimum pH value of 5.4 was obtained after 48 h. The pH value then increased again and after 96 h a pH value of 6.4 was measured. For the tripled phosphate conditions, the pH value decreased to 5.2 at 48 h and then even further to 4.9 after 72 h.

For the presented experiment, ammonium and phosphate concentrations were also measured. The ammonium consumption was similar for both approaches and the progression was comparable to the cultivation under standard conditions in Chapter 4.1. Therefore, the data were not included into Fig. 4-2. The phosphate consumption for the standard conditions was identical to the experiment discussed in Chapter 4.1. The phosphate concentration decreased strongly at the beginning of the cultivation and became limiting at 41 h (Fig. 4-2C). For the tripled phosphate approach, the phosphate concentration also decreased strongly during the first 40 h, but did not become limiting (Fig. 4-2F). The minimum of 270 mg L^{-1}, which is about one third of the initial tripled phosphate concentration, was determined after 96 h. Therefore, the phosphate requirement of the cells under standard conditions to grow under phosphate unlimited conditions is twice as high as the actual phosphate concentration in the medium.

For the standard conditions, all three overflow metabolites acetate, acetoin, and 2,3-butanediol were detectable in the fermentation broth at 41 h (Figs. 4-2C and F). The highest concentrations were determined between 90-96 h: 3.4 g L^{-1} acetate, 6.2 g L^{-1} acetoin, and 5.2 g L^{-1} 2,3-butanediol. For the tripled phosphate conditions, acetate was already measured at 24 h and the other two metabolites at 41 h. The highest concentrations were also measured at the end of the sampling time frame, between 96-120 h: 2.8 g L^{-1} acetate, 10.8 g L^{-1} acetoin, and 2.6 g L^{-1} 2,3-butanediol. For both conditions, roughly the same total concentration of by-products was formed, only the distribution of the three overflow metabolites was slightly different.

The tripled phosphate concentration in the medium did not show a positive influence on γ-PGA production. However, that does not mean that an increase of the phosphate concentration in general has to have a negative influence. The standard phosphate concentration leads to phosphate limitation of the cells during the first 48 h of cultivation. The results showed that for phosphate unlimited growth, the phosphate concentration in the standard medium E probably would have to be doubled. The optimum phosphate concentration for the γ-PGA production process can therefore possibly be found between the current and a doubled concentration.

4.3 Influence of additional citric acid

The reference cultivation in Chapter 4.1 showed that the maximum viscosity coincided with two events, a phosphate limitation and the depletion of the carbon source citric acid. The influence of a higher phosphate concentration was discussed in the chapter before. No positive influence on γ-PGA formation was observed. In this chapter, the results of an experiment are presented, in which the initial citric acid concentration was increased by 50 %, from 12 to 18 g L^{-1} (1.5x citric acid). In a second approach, the cultures started with the standard concentration of 12 g L^{-1} citric acid, and after 48 h, when citric acid was usually depleted, a citric acid pulse was applied, corresponding to additional 6 g L^{-1} (citric acid pulse). Figure 4-3 shows the experimental data of the two approaches with additional citric acid. For comparison, the OTR curves and the effective viscosity of the standard conditions are plotted in the same diagram. It was discussed in more detail in Chapter 4.1, that the respiration activities of cultures of the strain ATCC 9945 often show a divergence at some point of time. Therefore,

for all medium compositions which were investigated in this chapter, cultures under standard conditions were run in parallel as an internal reference. From these cultures, only OTR curves and effective viscosity are depicted in this chapter and also in the next chapter, to avoid showing and discussing very similar data repeatedly. All cultivation conditions were identical except for the different citric acid concentrations and the approaches were run in parallel. For each sampling point, two shake flasks were withdrawn from the shaker and not placed back. The shown offline values are the mean of the two flasks. Up to the time point of the citric acid pulse at 48 h, the same flasks were used for the sampling of the standard conditions and the conditions with the citric acid pulse.

The OTR curves of all three conditions were very similar (Figs. 4-3A and E). After the short lag phase followed by the exponential increase, all cultures reached the oxygen limitation at a level of approximately 11 mmol L^{-1} h^{-1} between 13-15 h. The OTR curves remained almost constant at this level until 240-250 h. For the 1.5x citric acid conditions, one of the OTR curves already decreased after about 160 h. For this RAMOS shake flask, a low final pH of 5.4 was measured. For the cultures with 1.5x citric acid, the OD_{600} reached 6.6 at 144 h and increased further to a final value of 10.6 at 310 h (Fig. 4-3A).Under conditions with a citric acid pulse, an OD_{600} of 8 was determined at 144 h (Fig. 4-3E). The OD_{600} remained at this level until the end of the cultivation.

For both, the standard conditions and the citric acid pulse, the maximum viscosity was determined at 48 h, namely 139 mPa s (Figs. 4-3E). For the cultures with 1.5x citric acid, a maximum viscosity of 273 mPa s was determined at 48 h (Fig. 4-3A). This shows a significant impact of citric acid on the γ-PGA production process. Interestingly, the citric acid pulse after 48 h did not affect the viscosity. The pulse was apparently set too late to positively influence the γ-PGA production. At 48 h, usually citric acid is depleted and the maximum viscosity is obtained. The process, which leads to the enzymatic degradation of the produced γ-PGA and, therefore, to the decreasing viscosity, is probably irreversible at this time point. Very recently, Mitsunaga et al. (2015) made the same observation. It was also noticed that a higher citric acid consumption was accompanied by a higher ammonium consumption. This coincidence was also determined for the experiments in this work (Figs. 4.3C and G). Mitsunaga et al. (2015) could increase the γ-PGA production by *B. licheniformis* ATCC 9945 by applying pulsed feeding of di-ammonium citrate after 24, 36, and 48 h.

Figures 4-3B and F show the carbon source consumption and pH values of the different conditions. As observed before, the three carbon sources glycerol, glutamic acid and citric acid were consumed in parallel. The initial citric acid concentration was depleted at 48 h. That means, the citric acid consumption rate was considerably higher for the cultures with the higher initial citric acid concentration. For the cultures with the citric acid pulse, a sample was taken directly before the pulse at 48 h and then again 5 h later. A low concentration of 0.7 g L^{-1} of citric acid, the remnants of the citric acid pulse, was measured at 53 h. However, a higher viscosity than for the sample at 48 h or even for the sample of the standard conditions at 53 h could not be determined. For both approaches, glycerol was depleted at 144 h and glutamic acid after 93 h. The cultures with 1.5x citric acid had an initial pH value of 7.6 (Fig. 4-3B). At 48 h a pH value of 5.5 was determined, which decreased even further to 5.3 at 72 h. At the end of the cultivation, a pH value of 9.4 was measured in the RAMOS shake flask with respiration activity until 310 h. For the cultures with the citric acid pulse, the pH minimum was obtained at 48 h with a pH value of 5.2 (Fig. 4-3F). After that, the pH value showed a sudden increase to 5.8 at 53 h, very probably due to the consumption of the added citric acid. The pH value increased further until the end of the cultivation, when a final value of 9.5 was measured. For the standard conditions, which also showed a long time of respiration activity, the final pH value was above 9, too.

Figure 4-3C shows the ammonium and phosphate consumption of the cultures with 1.5x citric acid. The phosphate concentration became limiting at 48 h and remained that way until at least 144 h. For the final sample, a concentration of 20 mg L^{-1} was determined. The ammonium concentration decreased strongly during the first 48 h. A concentration below 50 mg L^{-1} was measured at 48 h, which decreased below 30 mg L^{-1} until 53 h. The concentration remained at a low level until 93 h and then increased again. The final concentration at 310 h was 1060 mg L^{-1}. For the cultures with the citric acid pulse, a similar observation was made (Fig. 4-3G). Phosphate was depleted at 48 h. Ammonium decreased strongly during the first 48 h and became almost limiting at 53 h, when a concentration of only 20 mg L^{-1} was measured. Towards the end of the cultivation, the ammonium concentration also increased again. The final value was determined at 310 h with 1240 mg L^{-1}.

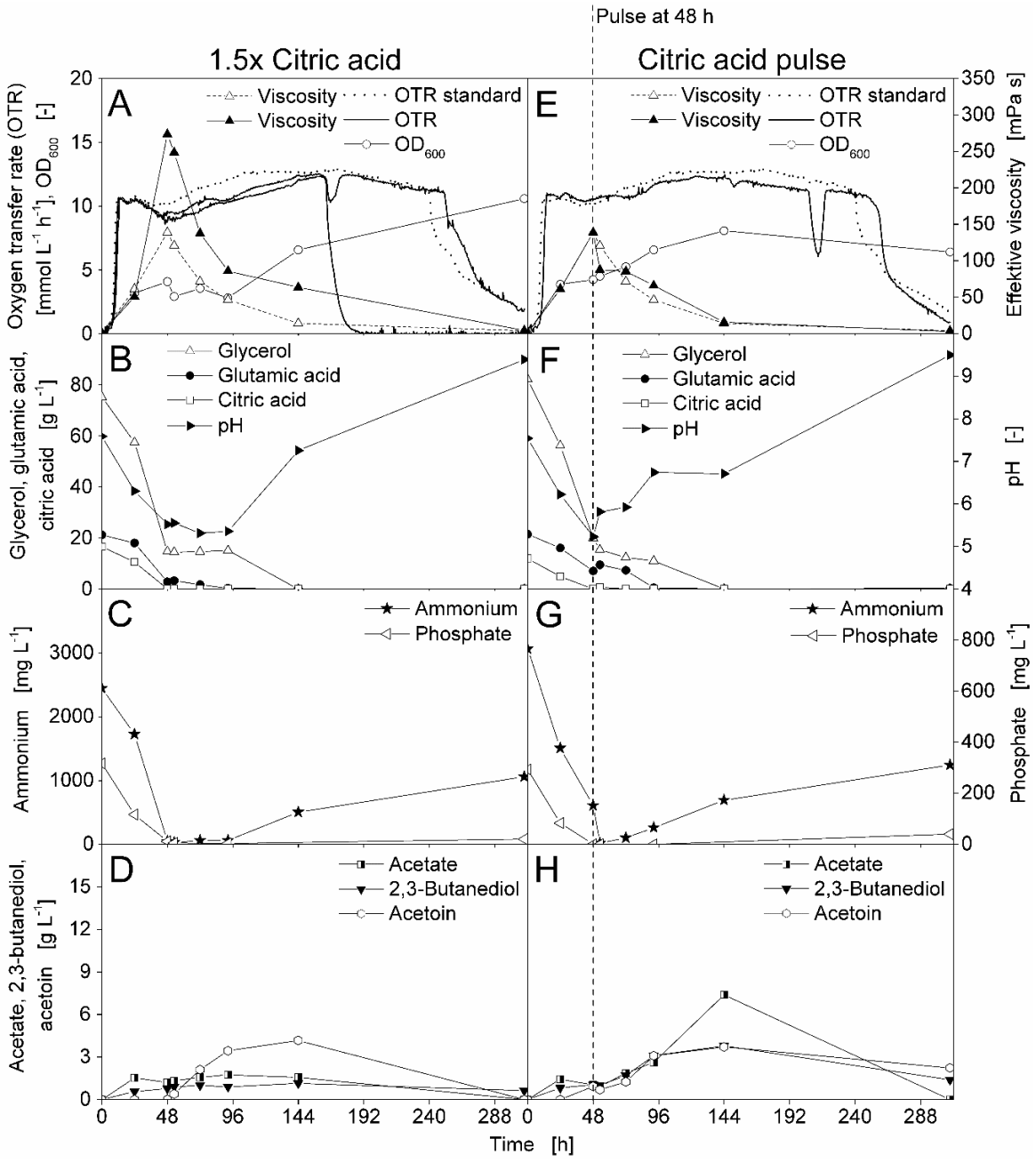

Figure 4-3 Impact of additional citric acid or a citric acid pulse (after 48 h) on γ-PGA production of *B. licheniformis* ATCC 9945

Oxygen transfer rates (OTR), optical density (OD_{600}), and effective viscosity are shown in **(A)** and **(E)**. Carbon source concentrations of glycerol, glutamic acid, and citric acid, as well as the pH value are shown in **(B)** and **(F)**. Ammonium and phosphate concentrations are presented in **(C)** and **(G)**. Overflow metabolite concentrations of acetate, acetoin, and 2,3-butanediol are shown in **(D**) and **(H)**. The dotted line indicates the time point of the citric acid pulse. Initial values: pH = 7.5-7.6, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 18 **(A-D)** or 12 plus 6 **(E-H)** g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from glycerol stock (0.4 % v/v).

The higher amount of citric acid apparently led to a higher consumption of ammonium, which in both cases resulted in very low ammonium concentrations. Due to the time spans between the sampling points, which were often 24 h, it cannot be excluded that an ammonium limitation occurred at some point of time.

The overflow metabolites acetate, acetoin, and 2,3-butanediol were formed in similar amounts for both conditions (Figs. 4-3D and H). For the conditions with 1.5x citric acid, acetate and 2,3-butanediol were already detectable at 24 h, acetoin only after 53 h. Here, the highest measured concentrations were 1.8 g L^{-1} acetate at 53 h, 1.1 g L^{-1} 2,3-butanediol and 4.2 g L^{-1} acetoin at 144 h. Acetate was completely consumed until the end of the cultivation, but 2.8 g L^{-1} acetoin and 0.6 g L^{-1} 2,3-butanediol were still detectable in the fermentation broth. For the citric acid pulse approach, the highest concentrations of overflow metabolites were 7.4 g L^{-1} acetate, 3.8 g L^{-1} 2,3-butanediol, and 3.7 g L^{-1} acetoin, all at 144 h. At the end of the cultivation, acetate had been completely consumed, but 2.2 g L^{-1} acetoin and 1.4 g L^{-1} 2,3-butanediol remained.

Comparing the OTR curves of the three approaches, a phenomenon can be seen which was observed for all cultivations with *B. licheniformis strain* ATCC 9945. Although the cultures enter into a state of oxygen limitation shortly after the beginning of the cultivation, the oxygen limited plateau is not exactly horizontal, as it would be expected during an oxygen limitation (Anderlei and Büchs, 2001). Rather, it was observed that the OTR, after entering the oxygen limitation, decreased slowly by about 2-3 mmol L^{-1} h^{-1}, with the minimum in the plateau often coinciding with the maximum viscosity (here: after 48 h). Then, the OTR slowly increased again by several mmol L^{-1} h^{-1}, even beyond the initial level of 11 mmol L^{-1} h^{-1} and often reached a maximum of about 13 mmol L^{-1} h^{-1}. These changes in the OTR level can be explained by two effects. First, the viscosity influences the OTR_{max} of an unbaffled shake flask system in such a way, that the OTR_{max} increases for moderate increases in viscosity from 1 to 10 mPa s, leading to an improved oxygen supply of the cells (Giese et al., 2014a). Up to 80 mPa s, the OTR_{max} is equal or not significantly lower than for waterlike viscosities. At higher viscosities up to 225 mPa s, the OTR_{max} is still at least 60 % of the OTR_{max} at 1 mPa s. Therefore, the OTR level is initially influenced by the significantly increasing viscosity during the first 48 h, which leads to the decrease in the OTR level. For the experiment depicted in Fig. 4-3, it can be clearly seen that the OTR of the approach with 1.5x citric acid decreases

more strongly than of the other two approaches. This correlates well with the much higher maximum viscosity which was observed for the 1.5x citric acid conditions. The increase in the OTR level can be partially attributed to the strongly decreasing viscosity after the maximum is reached. The second effect, which influences the OTR_{max} level, is the solubility of oxygen, which is, among other factors, influenced by the concentration of dissolved compounds in the fermentation broth. This concentration can be indirectly measured by determining the osmolality of the fermentation medium. For cultivations in medium E, the initial osmolality of the medium is approximately 1.7 osmol kg^{-1}. During the cultivation, due to the consumption of medium compounds by the microorganisms, especially of the carbon sources, the osmolality decreases. The final osmolality in the presented experiment was, for instance, about 0.7 osmol kg^{-1} (Fig. 7-4, appendix). This decrease in osmolality can lead to an increase in OTR_{max} and, therefore, to a slight increase in the OTR level under oxygen limited conditions.

The results of the presented experiment demonstrate a strong influence of citric acid on the maximum viscosity and, hence, on the γ-PGA production process. The increase of the initial citric acid concentration by 50 % led almost to a doubling of the maximum viscosity. However, the addition of 50 % of the initial concentration in form of a pulse at 48 h did not result in any change in the maximum viscosity. The pulse probably was set too late to have an effect, since at 48 h, citric acid was already depleted and the potentially irreversible enzymatic degradation process of the produced γ-PGA (indicated by the decreasing viscosity) had already started. The results showed also, that a higher citric acid concentration caused a higher ammonium consumption, resulting in very low ammonium concentrations and possibly also in an ammonium limitation during the cultivation. Since ammonium is important for both, cell growth and product formation, a limitation of ammonium would severely influence the γ-PGA production process. Therefore, in further experiments the influence of a higher citric acid concentration combined with a higher ammonium concentration was investigated. Moreover, the combination of a higher citric acid concentration with a higher phosphate concentration was tested. If the carbon source concentration is increased, also the demand of the cells for phosphate should be increasing.

4.4 Influence of additional citric acid and ammonium or phosphate

The results of the experiment presented in Chapter 4.3 showed a strong influence of citric acid on the maximum viscosity. The conditions with 1.5x of the initial citric acid concentration led to an almost doubled maximum viscosity compared to the standard conditions. The results showed also that a higher citric acid concentration led to a higher ammonium consumption, leading in turn probably to an ammonium limitation during the cultivation. Since ammonium is an important nutrient for cell growth and product formation, respectively, a limitation of ammonium would severely influence the γ-PGA production. In this chapter, the influence of a higher citric acid concentration combined with a higher ammonium concentration or a higher phosphate concentration was investigated. The combination of a higher citric acid and phosphate concentration was tested, because if the carbon source concentration is increased, also the demand for phosphate of the cells could be increasing. For both conditions, the initial citric acid concentration was increased by 50 %, from 12 to 18 g L^{-1} (1.5x citric acid), identical to the conditions presented in Chapter 4.3. Moreover, either the ammonium concentration was increased by 50 %, from 7 to 10.5 g L^{-1}, or the phosphate concentration was increased by 200 %, from 0.5 to 1.5 g L^{-1}. Figure 4-4 shows the comparison of the two approaches with 1.5x citric acid and 1.5x ammonium or 3x phosphate compared with the OTR curves and effective viscosity curves of the parallel standard conditions. All cultivation conditions were identical except for the different citric acid, ammonium, and phosphate concentrations and the three approaches were run in parallel. For each sampling point, two shake flasks were withdrawn from the shaker and not placed back. The shown offline values are the mean of the two flasks.

For this experiment, the duration of the respiration activities of the three conditions were quite different (Figs. 4-4A and E). After the short lag phase followed by the exponential increase, all cultures reached the oxygen limitation at a level of approximately 11 mmol L^{-1} h^{-1} between 11-15 h. The OTR curves of the standard condition remained almost constant at this level until 220 h, when they started to decrease. The final pH value for the two RAMOS shake flasks was 9.3. For the 1.5x citric acid/3x phosphate conditions, all three OTR curves already decreased after about 150 h and had final pH values of 5.4. For the 1.5x citric acid/ammonium conditions, the OTR curves decreased even earlier, already after about 50 h. They, too, had

low final pH values of 5.1. This clearly demonstrates again the coincidence of an early termination of respiration activity with low final pH values, which was already described in Chapter 4.1. For the approach with 1.5x citric acid/3x phosphate, the maximum OD_{600} of 12.5 was achieved at 190 h (Fig. 4-4A). For the 1.5x citric acid/ammonium conditions, the OD_{600} increased only to a value of 4.5 at 44 h and then remained at this level until the end of the experiment (Fig. 4-4E).

For all three conditions, the maximum viscosity was determined at 44 h: 158 mPa s for the standard conditions, 323 mPa s for 1.5x citric acid/3x phosphate, and 261 mPa s for the approach with 1.5x citric acid/ammonium (Figs. 4-4A and E). Thus, the conditions with additional citric acid showed again a significantly higher maximum viscosity than the standard conditions. Compared with the conditions with additional citric acid only (see Chapter 4.3; 273 mPa s), the obtained viscosities were higher for 1.5x citric acid/3x phosphate and in the same range for 1.5x citric acid/ammonium. The additional ammonium therefore had no further positive effect. Phosphate however showed a synergy effect with citric acid. The maximum viscosities of the standard conditions in both experiments were in the same range (158 mPa s here, compared to 139 mPa s in Chapter 4.3).

Figures 4-3B and F show carbon source consumption and pH values of the two different conditions. Also here, the three carbon sources glycerol, glutamic acid, and citric acid were consumed in parallel. Citric acid was depleted at 44 h. Again, the citric acid consumption rate was clearly higher for the cultures with higher initial citric acid and ammonium or phosphate concentration compared to the standard conditions. This could be observed already for the cultures with higher initial citric acid concentration only (Chapter 4.3). For the approach with 1.5x citric acid/3x phosphate, glycerol was depleted at 144 h. For the 1.5x citric acid/ammonium cultures, glycerol decreased to 33.6 g L^{-1} during the first 72 h and remained at this level until the end of the experiment. Due to the early termination of respiration activity of the cultures, glycerol was not completely consumed. The same was true for glutamic acid: at 44 h, the concentration had decreased to 6.2 g L^{-1} and afterwards remained at that level. For the conditions with 1.5x citric acid/3x phosphate, glutamic acid was already depleted at 70 h.

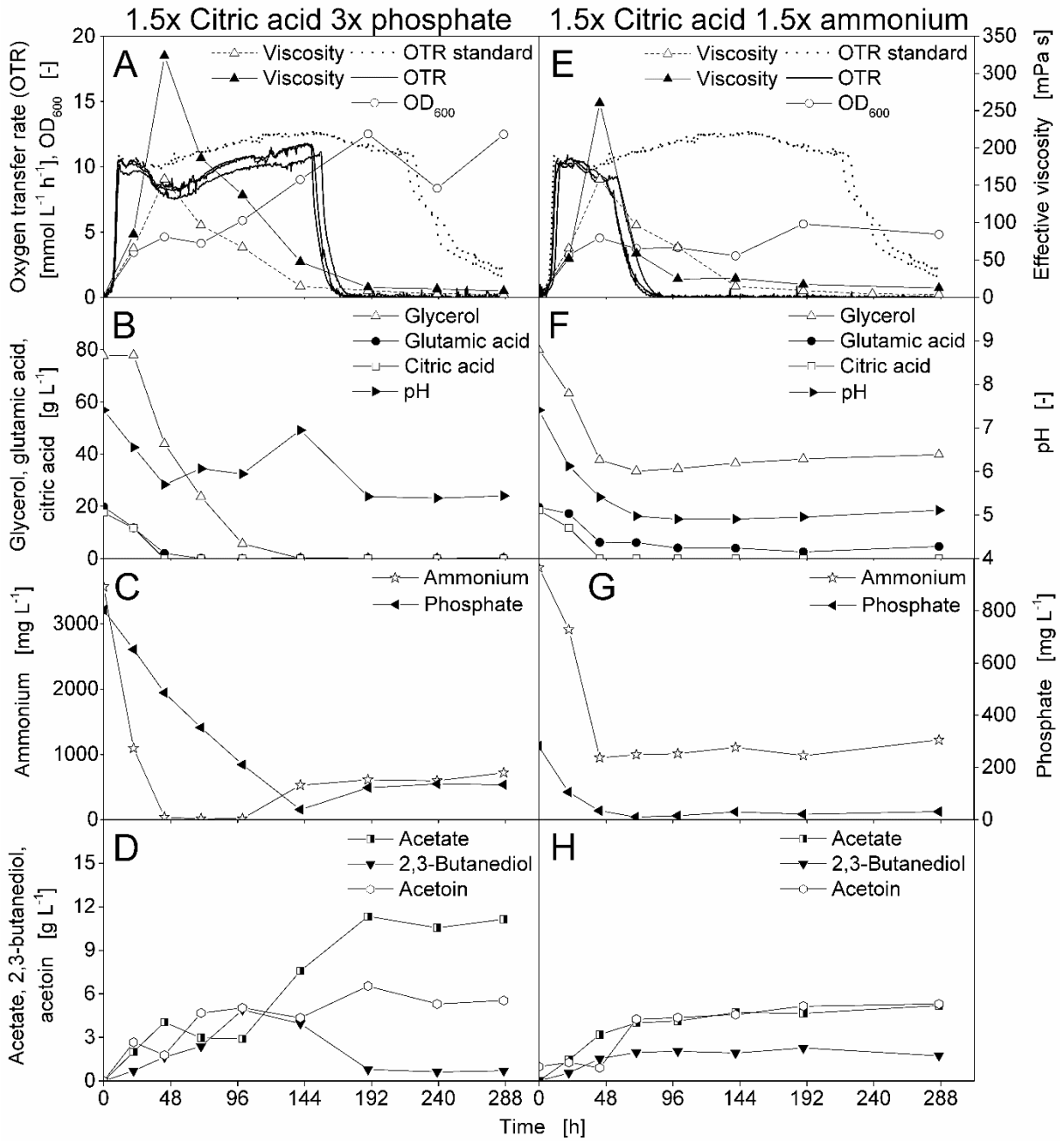

Figure 4-4 Impact of additional citric acid and phosphate or additional citric acid and ammonium on γ-PGA production of *B. licheniformis* ATCC 9945

Oxygen transfer rates (OTR), optical density (OD_{600}), and effective viscosity are shown in **(A)** and **(E)**. Carbon source concentrations of glycerol, glutamic acid, and citric acid, as well as the pH value are shown in **(B)** and **(F)**. Ammonium and phosphate concentrations are presented in **(C)** and **(G)**. Overflow metabolite concentrations of acetate, acetoin, and 2,3-butanediol are shown in **(D**) and **(H)**. Initial values: pH = 7.4, 76.3 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 18 g L^{-1} citric acid, 7 **(A-D)** or 10.5 **(E-H)** g L^{-1} NH_4Cl, 1.5 **(A-D)** or 0.5 **(E-H)** g L^{-1} K_2HPO_4. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from glycerol stock (0.4 % v/v).

The cultures with 1.5x citric acid/3x phosphate had an initial pH value of 7.4 (Fig. 4-4B). At 44 h, a pH value of 5.7 was determined, which (with one exception) remained at this level until the end of the cultivation. For the conditions with 1.5x citric acid/ammonium, the pH value decreased from 7.4 to 5.0 in the first 70 h and remained at this very low level, which correlates well with the early termination of respiration activity (Fig. 4-3E and F).

For the conditions with 1.5x citric acid/3x phosphate, ammonium and phosphate consumption were clearly higher than for cultures under standard conditions (Fig. 4-4C, compare with Chapter 4.1). At 44 h, the ammonium concentration became almost limiting. A concentration below 50 mg L^{-1} was measured. The concentration remained at this low level until about 100 h and then increased again. The final concentration at 287 h was 720 mg L^{-1}. The phosphate concentration decreased constantly from the initial value of 800 mg L^{-1} to only 40 mg L^{-1} at 141 h. This shows that the actual demand of phosphate is definitively above the standard concentration. The demand is even increased in case of 1.5x citric acid. Here, the additional phosphate, which guaranteed phosphate unlimited conditions, had a positive effect which resulted in a maximum viscosity even higher than for 1.5x citric acid only (Chapter 4.3). In case of the 1.5x citric acid/ammonium conditions, the ammonium concentration decreased by more than two-thirds during the first 44 h to 950 mg L^{-1} (Fig. 4-4G). The ammonium concentration then remained constant until the end of the experiment due to the early termination of respiration activity of the cultures. The phosphate concentration also decreased strongly and reached 10 mg L^{-1} at 70 h. This low level prevailed until the end. The increase of ammonium by 50 % successfully prevented an ammonium limitation for conditions with 1.5x citric acid. However, this increase of the ammonium concentration also led to a very early termination of respiration activity of the cultures after only 50 h. Due to the increased ammonium consumption, the pH value decreased very strongly and fell as low as 5.0 during the cultivation. The cultures apparently could not recover from this low pH value and, therefore, their respiration activity terminated.

Figures 4-4D and H show the formation of the overflow metabolites acetate, acetoin, and 2,3-butanediol. For both conditions, all three overflow metabolites were already detectable at 22 h. In case of 1.5x citric acid/3x phosphate, the highest measured concentrations were 11.3 g L^{-1} acetate and 6.5 g L^{-1} acetoin, both at 190 h, which marked also the end of the respiration activity of the cultures. Therefore, the concentration of these two by-products

remained at this level. The highest 2,3-butanediol concentration was 4.9 g L^{-1}, measured at 100 h. The concentration decreased to 0.8 g L^{-1} at 190 h and then remained at this level. For the conditions with 1.5x citric acid/ammonium, the concentrations of the three overflow metabolites increased, until the respiration activity of the cultures terminated, and remained at these levels. At 70 h, 4.0 g L^{-1} acetate, 2.0 g L^{-1} 2,3-butanediol, as well as 4.3 g L^{-1} acetoin were measured.

On the one hand, the presented experiment reconfirmed the impact of an increased citric acid concentration on the maximum viscosity and, thus, on the γ-PGA production process. On the other hand, the results showed that a parallel increase of the phosphate concentration had a further beneficial effect on the maximum viscosity while the increase of ammonium prevented a limitation but did not result in a higher maximum viscosity compared with additional citric acid only. A tripled phosphate concentration could prevent a phosphate limitation, even in case of 1.5x citric acid. For the standard citric acid concentration a tripled phosphate concentration (see Chapter 4.2) did not result in a higher viscosity. For conditions with 1.5x citric acid, however, the tripled phosphate concentration had a further positive effect. The increased ammonium concentration clearly prevented the decrease of the ammonium concentration into a limiting range. But the higher consumption of ammonium led to a strong decrease of the pH value, down to values no longer optimal for the growth of *B. licheniformis*. This led to a very early termination of the cultures' respiration activity.

4.5 Influence of different filling volumes

In the previous chapters, growth and production characteristics of *B. licheniformis* ATCC 9945 under standard conditions and in different medium compositions were described and discussed. Besides the influence of different medium compounds, also the impact of different filling volumes on growth and γ-PGA production was investigated. In this chapter, the results of cultivations with four different filling volumes are presented. The cultivation was conducted with standard mineral medium E, in 250 mL shake flasks, with a shaking frequency of 250 rpm, and a shaking diameter of 50 mm. The four different filling volumes were 10, 15, 30, and 61 mL. The odd-numbered value of 61 mL was chosen because this filling volume in a 250 mL shake flask achieves the same k_La value as 100 mL in a 500 mL shake flask (Seletzky et al., 2007).

Figure 4-5 shows the oxygen transfer rates, effective viscosities, and pH values for the four different filling volumes. In case of a filling volume of 10 mL, oxygen unlimited conditions were achieved (Fig. 4-5A), while the three other filling volumes resulted in oxygen limited conditions (Figs. 4-5B-D). After a lag-phase of approximately 10 h, all OTRs increased exponentially to a certain level. For a filling volume of 10 mL, the OTR increased to its maximum of 41 mmol L^{-1} h^{-1} after 25 h. Here, the OTR showed a clear peak after which it decreased slowly to a value of about 22 mmol L^{-1} h^{-1} at 65 h. The slow decrease does not indicate the depletion of a carbon source but is typically a hint for a second substrate limitation (Anderlei and Büchs, 2001). The limiting substrate is neither ammonium, which usually does not become limiting during cultivations (see Chapter 4.1), nor phosphate, which became limiting in this cultivation after about 48 h (data not shown). Interestingly, the limitation of phosphate is not indicated by the OTR. The limiting substrate could either be one of the metal trace elements present in the medium, e.g. Mg^{2+}, Mn^{2+}, Fe^{3+}, or Ca^{2+}, or a trace element which is not part of the standard medium composition, for instance Co^{+}, Zn^{2+}, or Cu^{2+}. Moreover, a limitation of sulfur is possible. Sulfur, in the form of sulfate (SO_4^{2-}), is only available in a low concentration as the counter ion of Mg^{2+} and Mn^{2+}.

The OTRs of the filling volumes of 15, 30, and 61 mL increased to 33, 20, and 11 mmol L^{-1} h^{-1}, respectively (Figs. 4-5B-D). At these levels, the OTRs entered into oxygen limited plateaus. For the filling volume of 15 mL, the OTR showed a plateau for about 15 h which indicates only a short phase of oxygen limitation. The OTR curves of the two parallel RAMOS flasks then diverged and showed similar but not identical progressions. After the plateau, both OTRs decreased: one to a value of 24 mmol L^{-1} h^{-1} at 63 h, the other to 29 mmol L^{-1} h^{-1} after 46 h. This OTR then increased again to 33 mmol L^{-1} h^{-1} after 60 h. After having reached the level of the oxygen limitation, the OTRs of the filling volumes of 30 and 61 mL remained at these plateaus until the end of the cultivations. At 65 h, the OTRs of the filling volumes of 10, 15, and 30 mL showed a sudden decrease which can be attributed to the depletion of glycerol (data not shown). In Fig. 4-5, this time point is indicated by the dotted line. For the filling volume of 61 mL, glycerol was only depleted at the end of the cultivation and no decrease in OTR was observed at 65 h.

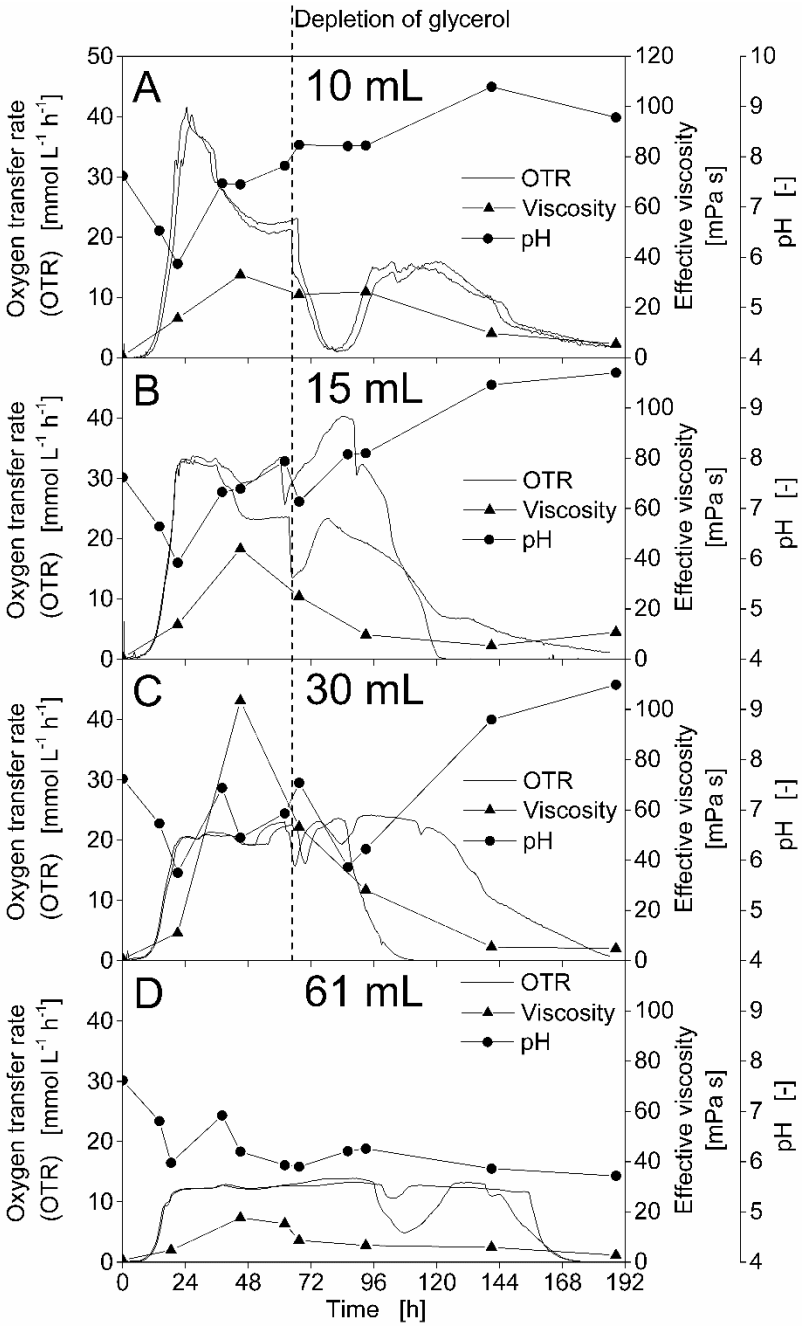

Figure 4-5 Trends of oxygen transfer rate, effective viscosity, and pH of *B. licheniformis* ATCC 9945 in shake flask cultivations with different filling volumes of standard mineral medium E

Filling volume 10 mL **(A)**. Filling volume 15 mL **(B)**. Filling volume 30 mL **(C)**. Filling volume 61 mL **(D)**. The dotted line indicates the time point of glycerol depletion for the filling volumes of 10, 15, and 30 mL. Initial values: pH = 7.6, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4. Cultivation conditions: T = 37 °C, 250 mL shake flasks, filling volume 10-61 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from glycerol stock (0.4 % v/v).

Between approximately 80 to 168 h, the cultures showed a second phase of respiration activity in which earlier produced overflow metabolites – acetate, acetoin, and 2,3-butanediol – are consumed. This second phase of respiration activity was most pronounced for a filling volume of 10 mL, but could also be identified for a filling volume of 15 mL (Figs. 4-5A and B). For a filling volume of 30 mL, only the very small drop in the OTR level at 65 h indicated the shift in the carbon source metabolism (Fig. 4-5C). And for 61 mL, the plateau of oxygen limitation masked the change between carbon sources completely (Fig. 4-5D). For the filling volumes of 15, 30, and 61 mL, the OTRs also did not indicate a limitation of a second substrate as did the OTR of the 10 mL filling volume. The time point when the limitation set in was hidden by the OTR plateaus due to the oxygen limitation. For a filling volume of 15 mL, possibly the slow decrease after the plateau at 35 h indicates that the cultures were under a second substrate limitation. While the two OTR curves of the filling volume of 10 mL showed a reproducible progression, the OTR curves of the other filling volumes diverged at some point. For the filling volume of 15 mL, the OTR curves deviated after 35 h, showing similar progressions but with different absolute values. One of the two OTR curves for the filling volume of 30 mL started to decrease early, after 85 h, the other decreased only after about 120 h. For the filling volume of 61 mL, both OTR curves showed a drop between 95 to 125 h: one OTR curve showed small drop by about 2-3 mmol L^{-1} h^{-1} for roughly 10 h while the other OTR curve showed a larger drop down to 5 mmol L^{-1} h^{-1} over a period of 30 h. The drops cannot be attributed to the depletion of glycerol, since this carbon source was only completely consumed after more than 140 h, as described above. Also, none of the other carbon sources or overflow metabolites is depleted at this time point. Since the decrease in OTR is not sudden and sharp, but rather slow and soft, it was probably caused by a low pH value from which both cultures were able to recover. However, both OTRs decreased relatively early, after 144 and 155 h, respectively. In case of the 61 mL filling volume, any carbon source depletion is hidden beneath the plateau of the oxygen limitation. Only the influence of a low pH value is visible.

For all four filling volumes, a decrease of the pH value from 7.6 to the minimum of 5.8 to 6.0 after 21 h was observed. This decrease coincided with the exponential increase of the OTRs. For the filling volumes of 10 and 15 ml, respectively, the pH value strongly increased again. At the end of the cultivation, pH values of 8.8 and 9.5 were measured (Figs. 4-5A and B). The pH value for the filling volume of 30 mL also increased after its minimum, but showed some fluctuations before increasing strongly towards the end of the cultivation. The final pH value

was above 9 (Fig. 4-5C). For the filling volume of 61 mL, the pH value exhibited an increase after its minimum to 6.9. Then, the pH value decreased again and remained at a level of approximately pH 6. The final pH value was 5.7 (Fig. 4-5D).

For all four approaches, the maximum effective viscosity was measured at 45 h. After the maximum was reached, a significant decrease was observed, resulting in low effective viscosities of 10 mPa s or less at the end of the cultivations. For the filling volumes of 10 to 30 mL, maximum effective viscosities of 33, 44, and 104 mPa s were measured (Figs. 4-5A, B, and C). For 61 mL, the maximum effective viscosity was only 18 mPa s (Fig. 4-5D).

The presented experiment shows the strong impact of the filling volume, and, therefore, of the maximum oxygen transfer capacity of the system, on respiration activity and γ-PGA production of *B. licheniformis* ATCC 9945. The cultures with the filling volumes of 10 and 15 mL were not, or only for a short time, oxygen limited during the cultivation. The cultures had high final pH values, which correlated with a high overall consumption of oxygen, reflected by the large integrals of the OTR curves. The OTR integrals are as large as the OTR integrals of the cultures under standard conditions described in Chapter 4.1, which exhibited a long time of respiration activity of 240 h or more. The cultures with the filling volumes of 30 and 61 mL were oxygen limited for almost the complete time of cultivation. For one of the RAMOS shake flasks of the 30 mL filling volume and for both RAMOS flasks of the 61 mL final pH values below 6 were measured. Here, the low final pH value correlated with a small OTR integral and a short time of respiration activity, respectively. Thus, the correlation between the final pH value and the size of the OTR integral is also valid for cultivation conditions with maximum oxygen transfer capacities other than the standard conditions. Apparently, the level of oxygen supply determines the pattern of carbon source consumption and by-product formation which in turn influences the progression of the pH value and, thereby, decides whether the cultures' respiration activity terminates early or not.

Interestingly, independent of the filling volume, the maximum effective viscosity was achieved at the same point of time. With increasing filling volume up to 30 mL and, hence, decreasing oxygen transfer capacity, an increase of the effective viscosity was observed. For the even higher filling volume of 61 mL, a significantly lower maximum effective viscosity was measured compared with 30 mL. When evaluating the different maximum viscosities, it

can be concluded that a maximum oxygen transfer capacity which results in a certain level of oxygen limitation is beneficial for γ-PGA production. However, if the maximum oxygen transfer capacity is too low, this has a negative impact on respiration activity and product formation. Here, the highest viscosity was obtained with a maximum oxygen transfer capacity of approximately 20 mmol L^{-1} h^{-1}.

It was observed already in 1954 that the filling volume of the shake flasks as well as the shaking frequency have an impact on γ-PGA production by *B. licheniformis* ATCC 9945 (Thorne et al., 1954). A lower filling volume and higher shaking frequency and, thus, a higher oxygen transfer capacity, resulted in increased γ-PGA yields. However, that the better yield is probably due to the higher oxygen supply was not mentioned in the publication. Possibly, the authors were not aware of this correlation. Moreover, with the applied filling volume of 150 mL in a 1000 mL shake flask and the shaking frequency of 100 rpm the oxygen transfer capacity was still as low as approximately 5 mmol L^{-1} h^{-1} (Seletzky et al., 2007). After all, the maximum oxygen transfer capacity of the standard conditions used in Chapter 4.1 was at least 11 mmol L^{-1} h^{-1}. And the results presented in this chapter demonstrated that apparently an even higher maximum oxygen transfer capacity of 20 mmol L^{-1} h^{-1} results in still higher viscosities. In more recent publications, cultivations for γ-PGA production in shake flasks are often conducted under "standard conditions", meaning 100 mL filling volume in 500 mL shake flasks at 250 rpm, or, also often, at lower shaking frequencies or in smaller shake flasks with a similar relative filling volume of 20 % (Bajaj et al., 2009; Birrer et al., 1994; Cao et al., 2010; Cromwick et al., 1994; Cromwick and Gross, 1995; Huang et al., 2011; Ito et al., 1996; Shih et al., 2002). From these information it can be concluded, therefore, that in many studies the achieved oxygen transfer capacities of the shake flask cultivations are equal to 11 mmol L^{-1} h^{-1} or lower.

For stirred tank bioreactors, several studies are published dealing with the influence of the oxygen transfer capacity on γ-PGA production of different *B. licheniformis* and *B. subtilis* strains (Cromwick et al., 1996; da Silva et al., 2014; Richard and Margaritis, 2003b). The general trend of the results is, that with a higher aeration rate and stirring rate, respectively, which result in a higher oxygen transfer capacity, the γ-PGA concentration increases. However, to deduce precise and practical values for the optimal cultivation conditions from these studies is difficult, since in each case different strains, medium compositions, aeration

rates and stirring speeds were applied. Additionally, no online data of e.g. the OTR or even the DOT are provided for an entire cultivation. To estimate the optimal oxygen transfer capacity for γ-PGA production from the published data is, therefore, hardly possible.

Up to date, no such investigation is known for shake flask cultivations. However, the data presented in this chapter and, also, the references dealing with stirred tank bioreactor cultivations, emphasize the importance of determining the optimal oxygen transfer capacity for the γ-PGA production process.

4.6 Summary

The *B. licheniformis* strain ATCC 9945 is a wildtype strain and known producer of the biopolymer γ-PGA. Its growth and γ-PGA production was characterized under standard conditions adapted from the literature. Subsequently, the impact of different medium compositions on γ-PGA formation was investigated. Finally, also the influence of different oxygen transfer capacities was studied by varying the filling volume of the shake flasks.

The characterization under standard conditions showed that at a certain time point during cultivation, the single cultures did not run in parallel anymore but instead diverged in their respiration and metabolic activity, respectively. Such a divergence of respiration activities of parallel cultures occurred independent of the medium composition. The deviation resulted in the early termination of respiration activity of some of the cultures. This phenomenon coincided with low final pH value of the respective flasks. As a possible reason for the divergence of the cultures the formation of subpopulations due to the initiation of sporulation was suggested.

The maximum effective viscosity was achieved after 48 h and, therefore, before the earliest diverging of the cultures, which was observed after 60 h. Thus, it can be concluded that the γ-PGA production phase is similar for all cultures and the deviation does not interfere with the production process. The maximum viscosity coincided with the depletion of the carbon source citric acid. Moreover, phosphate was limiting. The decrease in viscosity after its maximum indicated that an unknown factor apparently triggers the enzymatic degradation of γ-PGA. The influence of higher phosphate and citric acid concentrations on γ-PGA production was

investigated to determine whether the limitation of one of these compounds is important for the production process and triggers the enzymatic degradation. A non-limiting phosphate concentration did not improve the γ-PGA production. An increase of the initial citric acid concentration demonstrated a strong influence of citric acid on the maximum viscosity and, thus, on the γ-PGA production process. The increase of the initial citric acid concentration by 50 % led to a doubling of the maximum viscosity. The application of a citric acid pulse at 48 h, the time point of citric acid depletion, did not result in any change in the maximum viscosity. The higher citric acid concentration caused such a high ammonium consumption, that an ammonium limitation during cultivation could not be fully excluded.

Subsequently, the influence of a higher citric acid concentration combined with a higher ammonium concentration or a higher phosphate concentration was investigated. The experiments reconfirmed the impact of an increased citric acid concentration on the γ-PGA production process. A parallel increase of the phosphate concentration had a further beneficial effect on the maximum viscosity while the increase of ammonium prevented a limitation but did not result in a higher maximum viscosity compared with additional citric acid only. The higher consumption of ammonium led to a strong decrease of the pH value, down to values no longer optimal for the growth of *B. licheniformis*, which resulted in an early termination of respiration activity of all cultures.

The variation of the filling volume showed a strong impact of the maximum oxygen transfer capacity on respiration activity and γ-PGA production of *B. licheniformis* ATCC 9945. Low filling volumes and, thus, high maximum oxygen transfer capacities enabled oxygen unlimited conditions or oxygen limited conditions for only a short period of time. These conditions were beneficial for the reproducibility of the cultures and promoted a long period of respiration activity. In contrast, cultures with lower maximum oxygen transfer capacities, which resulted in long periods of oxygen limitation, showed often an early termination of respiration activity. Independent of the filling volume was the maximum effective viscosity achieved at the same point of time. With increasing filling volume and decreasing oxygen transfer capacity, an increase of the effective viscosity was observed. The highest viscosity was obtained with a maximum oxygen transfer capacity of 20 mmol L^{-1} h^{-1}. For the even lower maximum oxygen transfer capacity of the standard filling volume, a significantly lower maximum effective viscosity was measured. It can be concluded that a maximum oxygen transfer capacity which

results in a certain level of oxygen limitation is beneficial for γ-PGA production. However, if the maximum oxygen transfer capacity is too low, this has a negative impact on respiration activity and product formation.

The results showed that the trigger for the start of the enzymatic degradation is apparently neither the phosphate limitation nor the depletion of citric acid. The depletion or a lack of another nutrient such as a trace element could also initiate the degradation. The experiment with different filling volumes revealed that a second substrate limitation occurred before phosphate or citric acid were depleted. Therefore, in a subsequent experiment, also a supplementation with trace elements was investigated (Chapter 5.2).

Chapter 5

Investigation of poly(γ-glutamic acid) production via online determination of power input in shake flasks and the impact of strain and culture conditions

The previous chapter showed the characterization of the γ-PGA producing *B. licheniformis* strain ATCC 9945 in shake flasks cultivations under standard conditions from the literature. Additionally, different factors influencing the γ-PGA production process were investigated. These were different medium compositions and filling volumes, i.e. different levels of oxygen supply. The online measurement of the oxygen transfer rate was combined with thorough offline sampling to obtain intensive knowledge about metabolic activity and γ-PGA production of *B. licheniformis* ATCC 9945. In the present chapter, online power input measurement for shake flask cultivations is applied to follow γ-PGA production and degradation. The combination of the online signals of OTR and volumetric power input enables to continuously monitor respiration activity and product formation and, thereby, to easily investigate new strains, cultivation conditions, and medium compositions for their potential in γ-PGA production. In Chapter 5.1, the results of a cultivation of strain ATCC 9945 under standard conditions are presented. In Chapter 5.2, the results of an increased citric acid concentration and of a supplementation with trace elements on product formation and, thus, on the power input signal are shown. Chapter 5.3 presents the cultivation of another γ-PGA producing *B. licheniformis* strain, A1, under standard conditions. Strain A1 is originally a protease producing strain and was already introduced in Chapter 3. Chapter 5.4 shows a comparison of the strains ATCC 9945 and A1 in different medium compositions. Chapter 5.5

presents a correlation between offline viscosity and online power input. Chapter 5.6 closes with a summary. Part of the results in this chapter were obtained through the work of Julia Arndt (bachelor thesis).

An overview of the cultivation procedure, the different applied cultivation systems and the determined online and offline data for all experiments presented in this chapter is given in Figure 5-1. A detailed description of the applied techniques can be found in the materials and methods chapter.

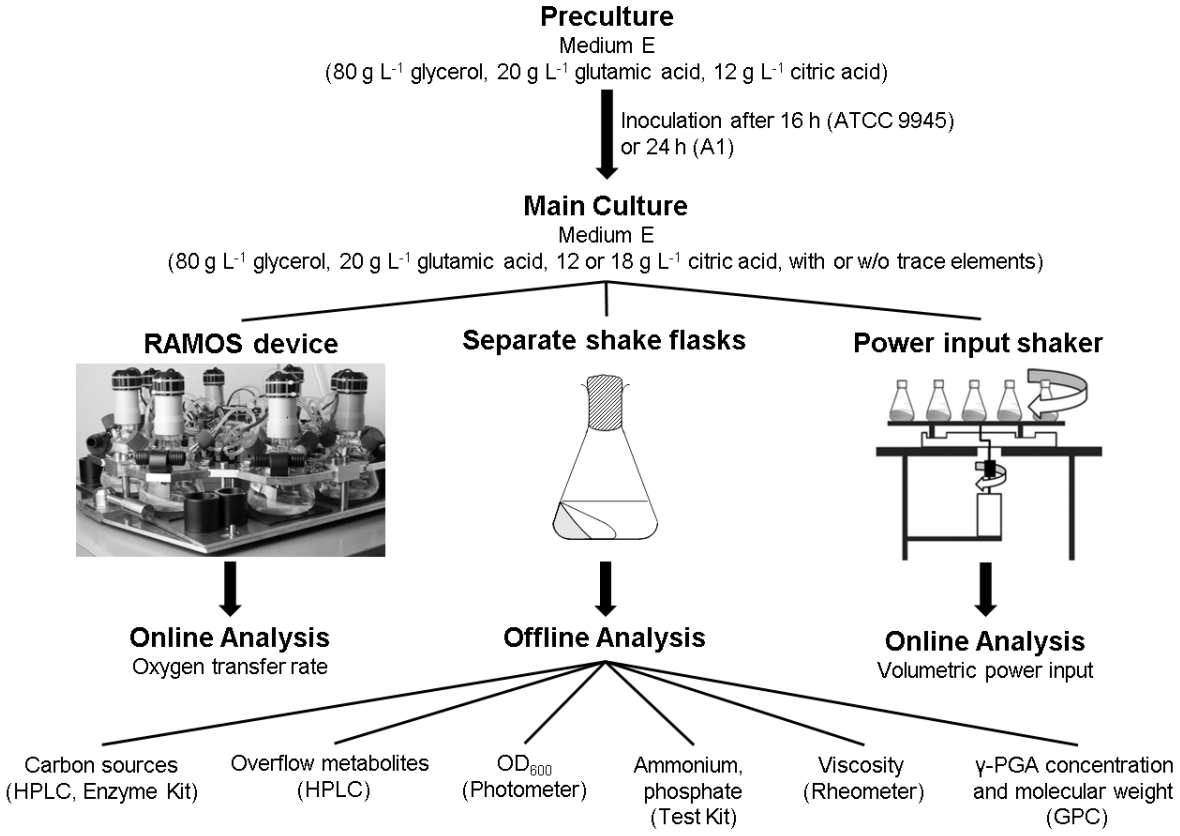

Figure 5-1 Overview of different cultivation steps, cultivation systems, and measured online and offline data

Both, pre-culture and main culture, were performed in mineral medium E. Pre-cultures were carried out in 500 mL shake flasks in a RAMOS (Respiration Activity MOnitoring System) device. The main culture was performed in 500 mL flasks in three cultivation systems in parallel: RAMOS device, power input shaker, and separate 500 mL shake flasks. The RAMOS device allowed for an online measurement of the oxygen transfer rate (OTR) of the culture. The power input shaker contained a torque sensor for online determination of the volumetric power input. Separate shake flasks were used for offline analysis.

5.1 Cultivation of *B. licheniformis* ATCC 9945 in standard mineral medium E

The strain *B. licheniformis* ATCC 9945 is one of the two strains used for the experiments depicted in this chapter to investigate γ-PGA production with online power input measurement. The strain ATCC 9945 is a known γ-PGA producer which is available from the *American Type Culture Collection*. Multiple publications are available about this strain (Birrer et al., 1994; Cromwick et al., 1996; Cromwick and Gross, 1995; Leonard et al., 1958; Thorne et al., 1954; Troy, 1973a, b). Figure 5-2 displays the results of a shake flask cultivation of *B. licheniformis* ATCC 9945 in standard mineral medium E (Leonard et al., 1958) with shaking conditions similar or identical to those often found published for production of γ-PGA (Birrer et al., 1994; Cromwick and Gross, 1995; Shih et al., 2002). Figure 5-2A shows the OTRs of six parallel RAMOS shake flasks. After a short lag-phase of 2 h, the OTRs increased exponentially up to 11 mmol L^{-1} h^{-1} and reached a horizontal plateau after 6 h which indicates an oxygen limitation (Anderlei and Büchs, 2001). After about 130 h, the OTRs of five of the six parallel flasks decreased, reaching 0 mmol L^{-1} h^{-1} after 155 h. The OTR of one of the flasks continued on the oxygen limited plateau and finally decreased after 225 h to reach 0 mmol L^{-1} h^{-1} after 290 h. For all six shake flasks, a smaller drop in the OTR to about 9 mmol L^{-1} h^{-1} is visible after 100 h. For the single flask which still showed respiration activity after 130 h, such drops in the OTR also occurred after 130 h and 193 h. The optical density increased constantly during cultivation to a maximum of 18 after 215 h, thereafter it declined to a final value of 13.5.

The online measured power input signal showed a strong increase during the first 24 h of cultivation (Fig. 5-2B). The sharp maximum of 2.1 kW m^{-3} was obtained after 26 h. Afterwards, the power input significantly decreased again and reached a level of 0.5-0.6 kW m^{-3} after 168 h, on which it remained until the end of cultivation. The offline measured effective viscosity correlated very well with the power input signal (Fig. 5-2B). It also showed a strong increase at the beginning of the cultivation. The highest viscosity of 103 mPa s was determined after 24 h. Parallel to the power input, the viscosity showed a distinct maximum after which it also decreased strongly, reaching values below 4 mPa s after 168 h. The viscosity remained at this low level until the end of the fermentation.

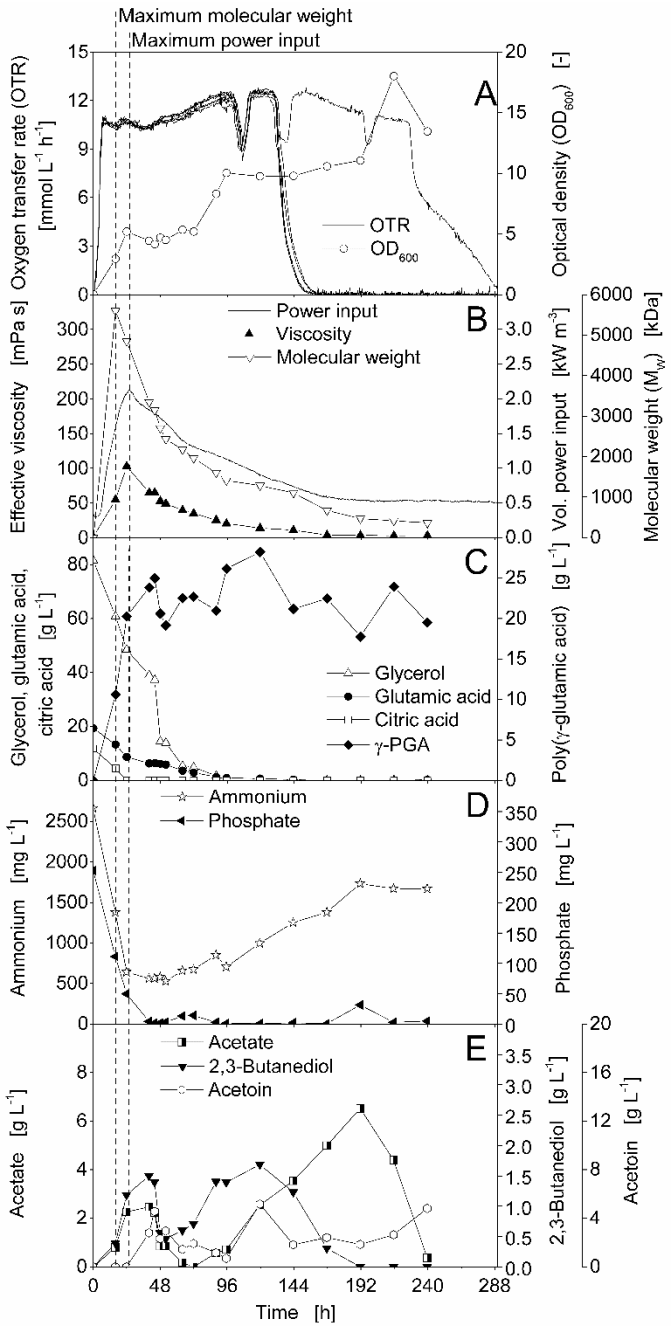

Figure 5-2 Growth and γ-PGA production of *B. licheniformis* ATCC 9945 in standard mineral medium E

Oxygen transfer rates (OTR) of six parallel flasks and optical density (OD_{600}) are shown in **(A)**. Effective viscosity, volumetric power input, and molecular weight of γ-PGA are presented in **(B)**. Carbon source concentrations of glycerol, glutamic acid, and citric acid, as well as γ-PGA concentration are shown in **(C)**. Ammonium and phosphate concentrations are presented in **(D)**. Overflow metabolite concentrations of acetate, acetoin, and 2,3-butanediol are shown in **(E)**. Initial values: pH = 7.4, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from pre-culture ($OD_{600,start}$ = 0.05).

Throughout this work, the determination of the effective viscosity was applied as a means for indirect γ-PGA measurement. The viscosity of a γ-PGA containing solution is influenced by the molecular weight as well as the concentration. Therefore, a method using gel permeation chromatography was established to enable the measurement of both parameters. With this technique, it became possible to quantify the changes in molecular weight and concentration of γ-PGA during cultivations, and to compare these with the trends of the online measured power input and the offline determined effective viscosity.

The maximum in γ-PGA molecular weight of 5600 kDa was determined at 16 h. Thereafter, it decreased significantly and reached a final value of 370 kDa after 240 h. After about 24 h, when the maximum viscosity and power input were measured, the molecular weight had already decreased to 4850 kDa. Interestingly, while the molecular weight decreased, the γ-PGA concentration still increased. At 16 h, the concentration was only 10.6 g L^{-1}, and after 24 h it had reached 20.3 g L^{-1}. The maximum concentration of 28.2 g L^{-1} was measured at 120 h. The final measured concentration after 240 h was 19.5 g L^{-1}.

Figure 5-2C displays the consumption of the three carbon sources glycerol, glutamic acid, and citric acid, as well as the γ-PGA concentration. All three carbon sources are consumed in parallel and not sequentially. Citric acid is first depleted after 24 h, followed by glutamic acid and glycerol, whose concentrations had decreased below 1 g L^{-1} at 96 h. The concentrations of ammonium and phosphate were also measured to determine whether the nitrogen or phosphate source might become limiting during cultivation. Starting at a concentration of 2670 mg L^{-1}, ammonium decreased strongly during the first 24 h to 640 mg L^{-1}. Between 24 and 48 h the concentration remained more or less constant and then increased again to about 1740 mg L^{-1} after 192 h. The final concentration was 1670 mg L^{-1} at 240 h. The initial phosphate concentration of 250 mg L^{-1} was depleted after 40 h and remained low until the end of the fermentation. During cultivation, the overflow metabolites acetate, acetoin, and 2,3-butanediol were formed and consumed later (Fig. 5-2E). Acetate showed the highest concentration with 6.5 g L^{-1} at 192 h. The highest concentrations of acetoin, 5.2 g L^{-1}, and 2,3-butanediol, 1.7 g L^{-1}, were obtained at 120 h.

With this experiment it could be shown that online measured power input and offline determined effective viscosity correlate very well. It is also obvious that the viscosity and,

therefore, also the power input, depends on both, molecular weight and concentration, of the produced γ-PGA. However, changes in the molecular weight seem to have a greater influence than changes in the concentration. For the biopolymer alginate it was shown before that a change in the molecular weight has a significant higher impact on the viscosity than an increase or decrease in concentration (Peña et al., 1997; Peña et al., 2007). The decrease in molecular weight of γ-PGA is very likely due to the secretion of γ-PGA specific depolymerases. From γ-PGA producing *Bacillus subtilis* strains, two degrading enzymes were reported, one acting in an endopeptidase-like fashion, cleaving the polymer into smaller oligomers (Ashiuchi et al., 2003b). These oligomers can be further cleaved by an exopeptidase into single glutamate monomers (Abe et al., 1997). Especially the activity of an endo-depolymerase should have a significant impact on molecular weight and, thus, on viscosity and power input. The activity of an exo-depolymerase is probably the limiting step in the degradation of the afore-produced γ-PGA to consumable glutamate monomers. After the depletion of the initial amount of glutamic acid, glutamic acid is not detected in the fermentation broth anymore. Thus, if any glutamic acid is released from the degradation of γ-PGA, it is instantly consumed. From the obtained experimental data, it cannot be stated without a doubt, that exo-depolymerases are secreted. However, the decrease in γ-PGA concentration between the maximum and the final value and the increase in ammonium concentration from about 48 h onwards suggests that glutamic acid is consumed as a carbon source thereby releasing excessive ammonium which is not needed as nitrogen source. The increase in ammonium concentration of about 1000 mg L^{-1} would correspond to the consumption of approximately 8 g L^{-1} glutamate, if none of the amino groups were to be used as nitrogen source. The values of the γ-PGA concentration fluctuates slightly towards the end, but a decrease in γ-PGA concentration of at least 5 g L^{-1} between the time point of maximum concentration and the end of the cultivation is reasonably to assume. This suggests, that not only a endo- but also an exo-depolymerase is expressed in an active form.

The enzymatic degradation of the produced γ-PGA starts, shown by the decrease in molecular weight, already after 16 h, when the carbon sources glycerol and glutamic acid are still present in significant concentrations (61 and 13 g L^{-1}, respectively). Therefore, the degradation has to be triggered by something else than the depletion of carbon source in general. Also an ammonium limitation as the trigger can be excluded, since the concentration never decreased below 500 mg L^{-1}. However, both the citric acid and the phosphate concentration were already

quite low at this time. Therefore, the influence of a higher citric acid and a higher phosphate concentration on γ-PGA formation was investigated. An increase of the phosphate concentration by the factor three showed no influence (see Chapter 4.2). The phosphate limitation thus seems to have neither a negative nor a positive influence on γ-PGA production under standard conditions. An influence of a higher initial citric acid concentration on the effective viscosity was already demonstrated (Chapter 4.3). In the next chapter, the influence of a higher citric acid concentration regarding its impact not only on viscosity, but also on power input, γ-PGA molecular weight, as well as the concentration is discussed.

5.2 Cultivation of *B licheniformis* ATCC 9945 in medium E with 1.5x citric acid or with trace elements

Figure 5-3A-E shows a cultivation of *B. licheniformis* ATCC 9945 in mineral medium E with 1.5x citric acid compared to the standard mineral medium E. The citric acid concentration was increased to investigate its influence on time point and absolute maximal values of γ-PGA molecular weight and concentration. In this experiment, the strain was cultivated for only 192 h compared to 330 h for the standard approach (Fig. 5-2), since the standard experiment showed that the interesting γ-PGA production and degradation phase takes place in the first approximately 96 to 120 h. Similar to the standard approach, the OTRs of the five parallel cultivated RAMOS shake flasks show almost no lag-phase followed by an exponential increase to 11 mmol L^{-1} h^{-1} (Fig. 5-3A). From this level, the OTRs continued as a horizontal plateau, indicating the oxygen limitation which was already observed for the standard approach. Between 140 to 155 h, three of the five OTRs started to decrease and reached a value of 0 mmol L^{-1} h^{-1} at 192 h. The other two OTRs showed only a drop to 8.5 and 6 mmol L^{-1} h^{-1}, respectively. Apparently, the cultures in these flasks recovered and continued to show respiration activity until the experiment was terminated. The final pH values of the cultures in the RAMOS flasks correlate with this observation. For the three flasks which showed an earlier termination of the respiration activity, a pH value of 5.6 was measured while the cultures with continued respiration activity had pH values of 7 and above. The optical density increased steadily from 0.05 up to a value of 11.6 at 144 h where it remained constant. Interestingly, with additional citric acid, a higher maximum viscosity, and correlating with it, a higher volumetric power input was measured. The maximum was also shifted slightly to a

later time point between 28 and 44 h (Fig. 5-3 B). The maximum viscosity was 178 mPa s, which is significantly higher than for the standard approach. The highest power input of 2.6 kW m^{-3} was determined after 36 h. The highest molecular weight of 5300 kDa was measured for the sample after 20 h. At the same time, a γ-PGA concentration of 11.4 g L^{-1} was determined. From 20 h onwards, the molecular weight decreased steadily throughout the cultivation to a final value of 250 kDa at 192 h. On the contrary, the γ-PGA concentration further increased to a maximum of 26 g L^{-1} at 52 h. The γ-PGA concentration remained almost constant at this level for about 24 h before it decreased. When the experiment was terminated after 192 h, a concentration of 10.5 g L^{-1} was measured.

Despite the increase of the initial concentration, citric acid was still the first carbon source which was depleted, however, slightly later than in the standard cultivation. Glutamic acid was depleted after 96 h and glycerol after 120 h. With a higher citric acid concentration in the medium, more ammonium was consumed than in the standard approach. A concentration of only 260 mg L^{-1} was measured after 44 h which is less than half of the lowest concentration determined in the standard cultivation (Fig. 5-2/3D). Yet, also in this experiment, an increase of the ammonium concentration was observed after the minimum value. The phosphate consumption was very similar to the standard cultivation. The overflow metabolites acetate, acetoin, and 2,3-butanediol were produced with maximum concentrations of 8.2, 16.2, and 3.4 g L^{-1}, respectively. Acetate production was in the same order of magnitude as for the standard cultivation. However, acetoin and 2,3-butanediol formation was significantly higher. An increase in the formation of these two metabolites is especially striking between 76 to 120 h, the same time frame, in which the γ-PGA concentration decreased. Probably the consumption of glutamate from γ-PGA induced the higher formation of reduced overflow metabolites. Since, under oxygen limited conditions, higher concentrations of reducing cofactors (NADH) in their reduced form accumulate, the formation of reduced overflow metabolites from more oxidized substrates can be used to re-oxidize the cofactors.

The increase of citric acid by 50 % (w/v) led to a significant increase in effective viscosity and volumetric power input. An increase was also observed for the maximum molecular weight, but not for the maximum concentration of γ-PGA. This demonstrates, that differences in the γ-PGA production, here, the different molecular weight, due to another medium composition can be detected with online power input measurement.

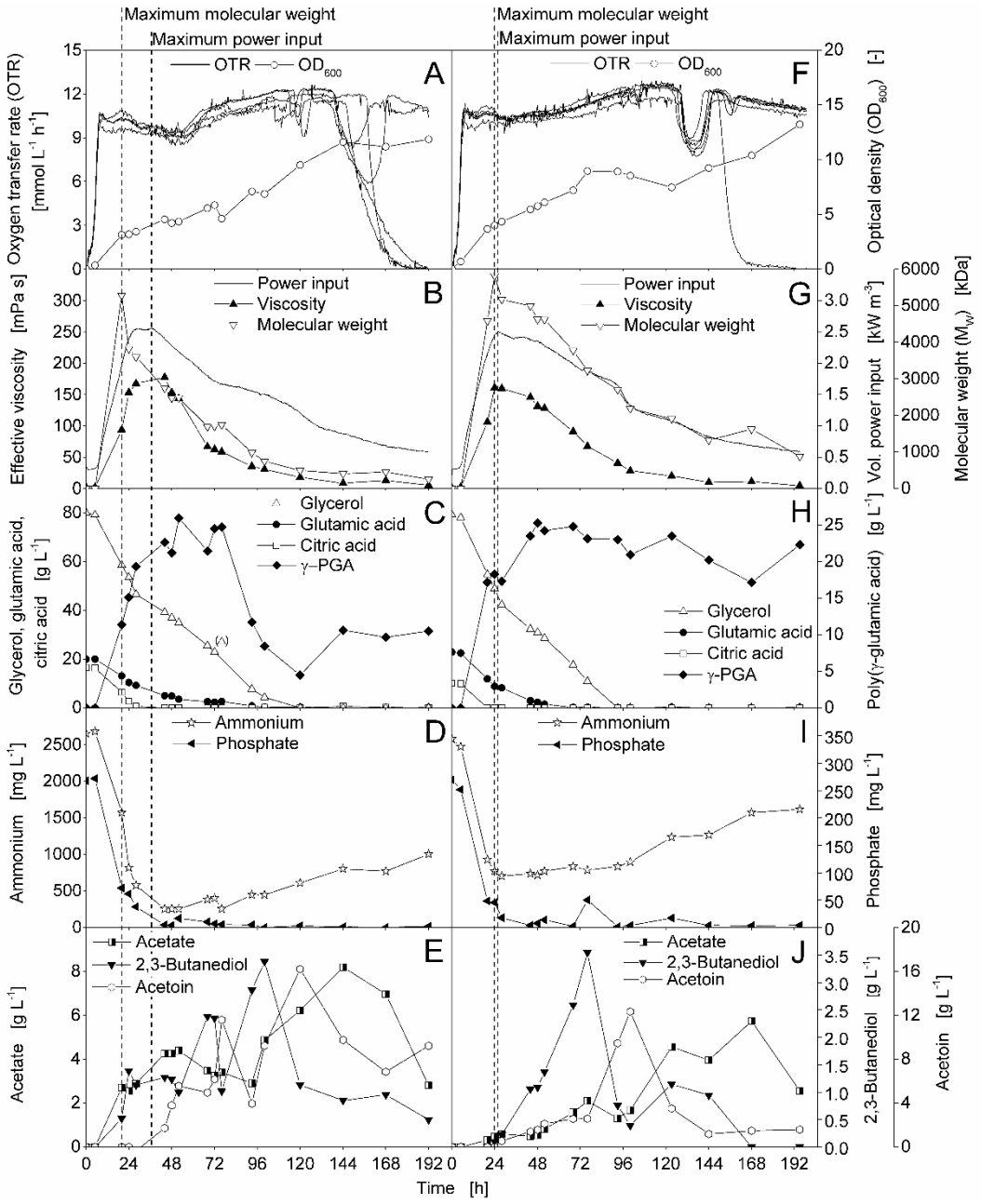

Figure 5-3 Impact of additional citric acid or trace elements on γ-PGA production of *B. licheniformis* ATCC 9945

Oxygen transfer rates (OTR) of five **(A)** and six **(F)** parallel flasks and optical density (OD_{600}), **(A)** and **(F)**. Effective viscosity, power input, and molecular weight of γ-PGA, **(B)** and **(G)**. Carbon source concentrations of glycerol, glutamic acid, and citric acid, as well as γ-PGA concentration, **(C)** and **(H)**. Ammonium and phosphate concentrations, **(D)** and **(I)**. Overflow metabolite concentrations of acetate, acetoin, and 2,3-butanediol, **(E**) and **(J)**. Initial values: pH = 7.4, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 18 **(A-E)** or 12 g L^{-1} **(F-J)** citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4, only **F-J**: trace elements solution (composition see Chapter 2.4). Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from pre-culture ($OD_{600,start}$ = 0.05).

Figure 5-3F-J shows a cultivation of *B. licheniformis* ATCC 9945 in mineral medium E with trace elements. Trace elements were supplemented to investigate whether the medium lacks certain micronutrients which are important for the γ-PGA production or if the lack of certain elements is involved in triggering the enzymatic γ-PGA degradation. In Chapter 4.5, the OTR curves of the cultures with only 10 mL filling volume, which were oxygen unlimited, depicted the typical shape of a limitation of a second substrate. In general, the OTRs curves of the six parallel cultivated RAMOS shake flasks displayed the same overall progression as the OTR curves from the experiments discussed above. Yet, the single OTRs seemed to run more in parallel. All flasks show a drop in the OTR between 125 and 145 h. The OTR of only one flask decreased completely after about 150 h while all other flasks showed continuous respiration activity until the experiment was terminated at 192 h (Fig. 5-3F). The maximal OD of 13.2 was measured after 192 h, which is slightly higher than for the other two conditions at the same time point.

The maximum viscosity was 161 mPa s, compared to 103 mPa s in the medium without trace elements. The maximum was measured after the same time as in the standard cultivation, namely after 24 h. The maximum power input was 2.5 kW m^{-3} at 26 h (Fig. 5-3G). The highest molecular weight of 5800 kDa was determined at 24 h and, therefore, slightly later than in the standard cultivation. The γ-PGA concentration was 18.3 g L^{-1} at this point and increased to a maximum of 25.3 g L^{-1} at 48 h. Similar to the standard cultivation and different to the experiment with 1.5x citric acid, the γ-PGA concentration afterwards decreased only slightly. The carbon sources citric acid, glutamic acid, and glycerol were depleted after 20, 68, and 93 h, respectively, which is similar to the standard approach regarding citric acid and glycerol. Glutamic acid was depleted approximately 24 h sooner in the medium with trace elements compared to the standard medium. Ammonium and phosphate consumptions were similar to the standard cultivation. After 28 h, a minimum of 710 mg L^{-1} ammonium was determined. The ammonium concentration remained at this level for about 24 h and then increased again to a final value of 1620 mg L^{-1}. The initial phosphate concentration was depleted at 44 h. The overflow metabolites acetate, acetoin, and 2,3-butanediol were produced in maximum concentrations of 5.7, 12.3, and 3.6 g L^{-1}. Acetate production was similar to the standard approach, but more acetoin and 2,3-butanediol were formed. This is similar to the cultivation with 1.5x citric acid.

The simple addition of trace elements such as cobalt, zinc, nickel, and copper resulted in a distinctly higher effective viscosity and volumetric power input. This suggests that in standard mineral medium E, as originally published by Leonard et al. (1958), some trace elements are missing to support unlimited growth and production. Leonard et al. also investigated the influence of cobalt and zinc on γ-PGA production and cell viability of *B. licheniformis* ATCC 9945. They found that varying concentrations of both ions influenced the D- to L-glutamate isomer ratio in the produced γ-PGA but did not increase γ-PGA concentration or cell viability. Therefore, they didn't include copper and zinc in their final medium composition. However, they also stated that although a requirement such as for zinc could not be confirmed in their experiments, they could not rule out that sufficient zinc was already provided by contamination of other medium ingredients. Thus, either other trace elements are limiting in medium E, for instance copper, which was not tested by Leonard et al.; or zinc and cobalt were indeed provided by contamination of other medium ingredients in their experiments (Leonard et al., 1958). It has to be kept in mind, that five decades ago, medium compounds were probably not produced with such a level of purity as today and, therefore, might have contained more trace elements. More recently, it was demonstrated that zinc boosts the activity of the γ-PGA synthesis complex *in vitro* (Ashiuchi et al., 2004). Therefore, a supplementation with this trace element might also increase γ-PGA production in *vivo*.

5.3 Cultivation of *B. licheniformis* A1 in standard mineral medium E

A second γ-PGA producing strain, *B. licheniformis* A1, was cultivated in standard mineral medium E (with kanamycin) to investigate how different strains vary in their growth and production behavior and if the differences can be detected with the online signals of OTR and volumetric power input. Figure 5-4 shows the results of the cultivation of strain A1. Identical to strain ATCC 9945, the strain A1 shows exponential growth after a very short lag-phase. Upon reaching an OTR level of 11 mmol L^{-1} h^{-1}, the cultures entered into an oxygen limitation indicated by the constant OTR level. Between 25 to 55 h, the OTRs of all six parallel RAMOS flasks showed a drop to about 4 mmol L^{-1} h^{-1}. The OTR plateau then remained constant until about 130 h when the OTRs show another drop, also down to 4 mmol L^{-1} h^{-1}. After 176 h, the OTRs started to decline slowly and reached 0 mmol L^{-1} h^{-1} after 240 h. The progression of the

OD correlates with the OTR. In general, the OD is increasing during the cultivation, but parallel to the drops in OTR also drops in optical density were observed. The highest OD of 9.2 was determined after 120 h. In this experiment, strain A1 showed a significantly lower viscosity, correlating with a lower volumetric power input, than strain ATCC 9945 under the same conditions. A maximum effective viscosity and power input of only 35.4 mPa s and 1.4 kW m^{-3} were determined at 24 h, respectively. The molecular weight of γ-PGA was with 4500 kDa also maximal at this point of time. The γ-PGA concentration was 8.6 g L^{-1} at 24 h, increased to its maximum of 13.7 g L^{-1} at 78 h, and decreased to 5.1 g L^{-1} until the end of the cultivation. Also, the carbon source consumption was different compared with strain ATCC 9945. Glycerol and glutamic acid were both depleted after 120 h. Citric acid, however, was only slowly consumed and at the end of the experiment, 1.4 g L^{-1} were still measured. This is in clear contrast to strain ATCC 9945, where in all experiments citric acid was the first carbon source to be depleted and also was a carbon source which had a measurable impact on γ-PGA production. Overflow formation of acetate and acetoin also differed between the two strains. Acetate was only formed towards the end of the cultivation with a concentration of 2.1 g L^{-1} measured at 246 h. The highest acetoin concentration of 16.1 g L^{-1} was measured at 120 h which correlates with the time point of glycerol and glutamic acid depletion. Part of the acetoin was consumed again and a final concentration of 6.4 g L^{-1} was obtained. However, 2,3-butanediol formation was in the same order of magnitude for both strains. A maximum concentration of 2.2 g L^{-1} was determined after 72 h. The formed 2,3-butanediol was completely consumed until the end of the cultivation. The strains also differed in their ammonium consumption. Strain ATCC 9945 showed a correlation between minimal ammonium concentration and maximal viscosity and power input, followed by an increase of ammonium towards the end of the fermentation. In contrast, strain A1 showed a constant decrease in ammonium concentration throughput the cultivation. A final value of 1180 mg L^{-1} was measured after 246 h.

This experiment shows how different two γ-PGA producing *B. licheniformis* strains can behave concerning growth and product formation. Yet, it also demonstrated that the differences between the strains can be detected using online measurement of OTR and volumetric power input as signals for respiration activity and product formation, respectively.

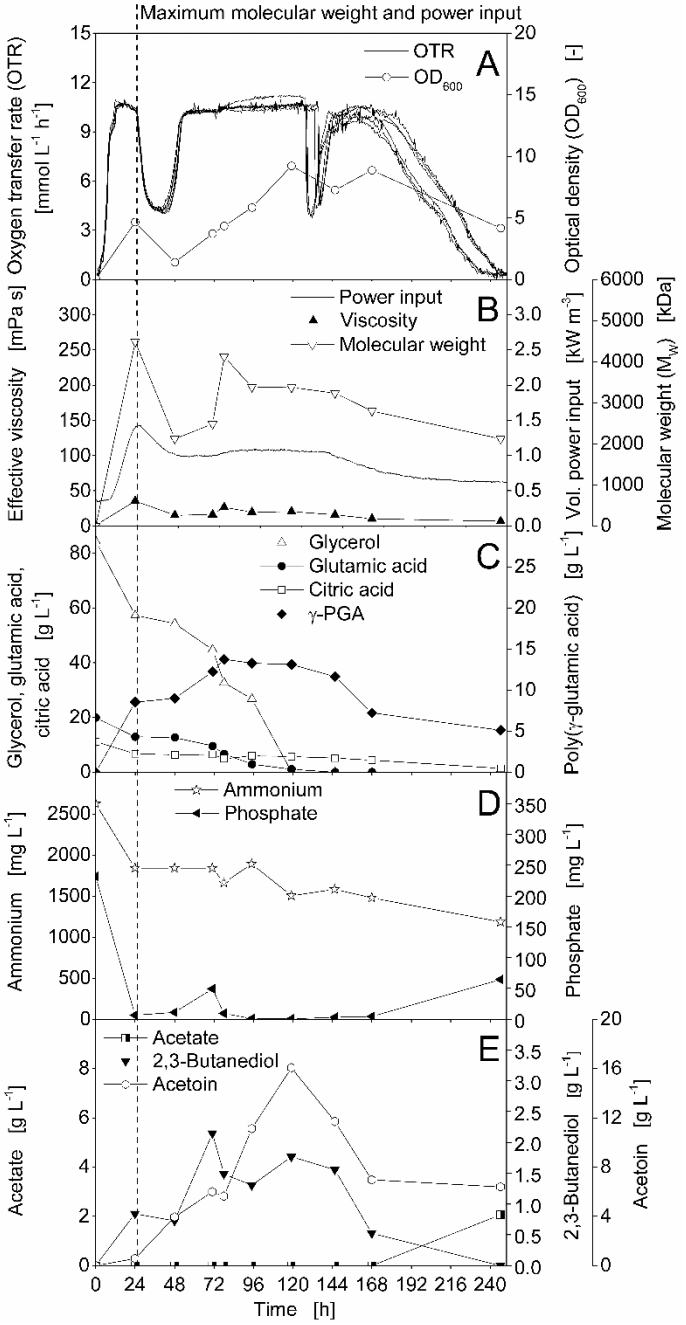

Figure 5-4 Growth and γ-PGA production of *B. licheniformis* A1 in standard mineral medium E

Oxygen transfer rates (OTR) of six parallel flasks as well as the optical density (OD_{600}) are shown in **(A)**. Effective viscosity, volumetric power input, and molecular weight of γ-PGA are presented in **(B)**. Carbon source concentrations of glycerol, glutamic acid, and citric acid, as well as γ-PGA concentration are shown in **(C)**. Ammonium and phosphate concentrations are presented in **(D)**. Overflow metabolite concentrations of acetate, acetoin, and 2,3-butanediol are shown in **(E)**. Initial values: pH = 7.4, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4, 50 mg L^{-1} kanamycin. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from pre-culture ($OD_{600,start}$ = 0.05).

5.4 Comparison of volumetric power input and effective viscosity of *B. licheniformis* ATCC 9945 and A1 in different variations of medium E

The strain A1 of *B. licheniformis* was not only characterized under standard conditions in mineral medium E, but was also investigated in different medium compositions. The supplementation with trace elements had shown a positive effect on γ-PGA production of strain ATCC 9945 (Chapter 5.2). Also, the γ-PGA production of strain A1 under standard conditions was considerably lower than that of ATCC 9945 (Chapter 5.3). Therefore, the supplementation of trace elements was investigated to determine whether the lower γ-PGA production of strain A1 is due to a limitation of trace elements. Strain A1 was usually cultivated with the antibiotic kanamycin, since it carries a plasmid with the respective resistance gene. However, experiments with this strain conducted during this work suggested that the antibiotic might have a negative influence on the γ-PGA production. Therefore, additional to a supplementation with trace elements also a cultivation without kanamycin was conducted to determine whether kanamycin has an influence on the γ-PGA production of strain A1. Figure 5-5 shows effective viscosity and volumetric power input measurements for different cultivations of *B. licheniformis* A1: in standard medium E with kanamycin, in medium E with trace elements and kanamycin, and in standard medium E without kanamycin. For comparison, the results of strain ATCC 9945 in standard medium E are also depicted.

Figure 5-5A shows the volumetric power input signals. For all cultivations, a (local) maximum at about 24 h was observed. Interestingly, for strain A1 in medium E with trace elements and in medium E without kanamycin, the actual maximum power input was measured much later in the cultivation, namely after 108 and 95 h, respectively. This progression is also reflected in the data of the offline measured viscosity (Fig. 5-5B). For the cultivation of strain A1 in standard medium E without kanamycin the highest effective viscosity and volumetric power input of all experiments was determined, which were 297 mPa s and 3.3 kW m^{-3}.

Obviously, in case of strain A1 the antibiotic kanamycin has a strong impact on γ-PGA production despite the strain's genetic resistance.

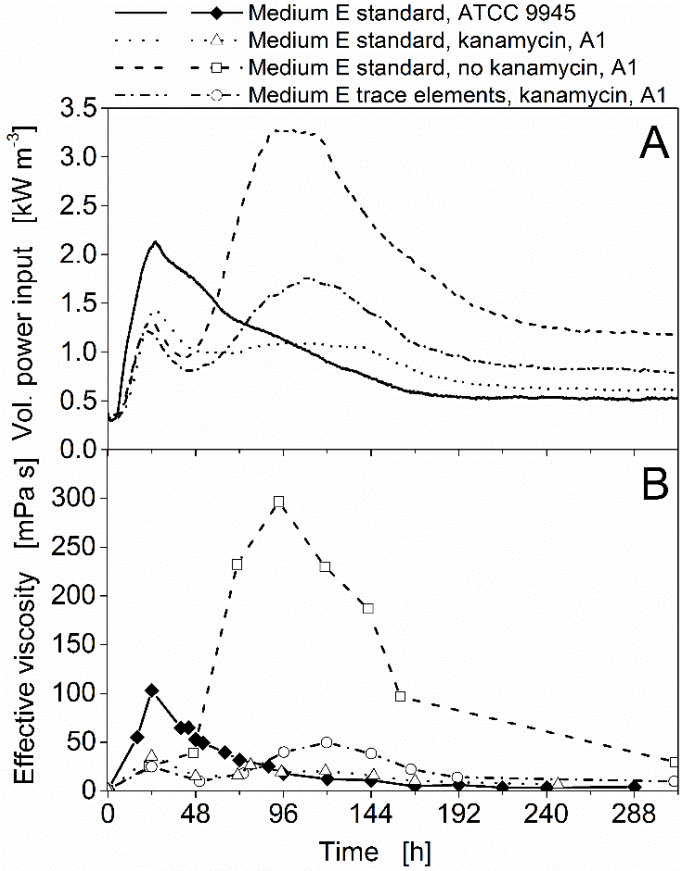

Figure 5-5 Comparison of γ-PGA production of *B. licheniformis* A1 and ATCC 9945 in different mineral medium E compositions

Online measured volumetric power input **(A)**. Offline measured effective viscosity **(B)**. Initial values: pH = 7.4, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4, with or w/o trace elements solution (composition see Chapter 2.4) and 50 mg L^{-1} kanamycin. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from pre-culture ($OD_{600,start}$ = 0.05).

5.5 Correlation between effective viscosity and volumetric power input

For shake flask cultivations, a correlation exists between effective viscosity and volumetric power input. With increasing viscosity the power input also increases and vice versa (Büchs et al., 2000b). This fact was also demonstrated by the experimental results shown in the previous chapter. Usually, the online power input signal can be used to calculate the effective viscosity (Büchs et al., 2000b). However, in case of the experiments shown in Chapters 5.1-5.4, it became obvious after the experiments had been performed, that the absolute torque signal, given by the torque sensor in the shaker drive, was not correct. Due to this, much lower viscosity values were calculated from the power input signal compared to the offline measured viscosity values from the rheometer measurements. To verify whether the general correlation between effective viscosity and power input was still intact, a fit equation was calculated from all viscosity and power input measurement data presented in Chapters 5.1-5.4. The results of this fit are shown in Fig. 5-6. From six experiments with different medium compositions and two different strains, 81 data points were used for the fit equation. The data point in brackets was omitted in the calculation (Fig. 5-6). As can be seen from the coefficient of determination R^2, which is 0.95, the fit equation fit the data very well. Subsequently, the determined fit equation was used to calculate an online effective viscosity from the online measured power input signal for the experiment described in Chapter 5.1.

Figure 5-7A depicts the offline measured effective viscosity from the rheometer measurements and the calculated online viscosity which was determined using the fit equation from Fig. 5-6. The very high correlation between measured and calculated viscosity data shows that the alternatively determined fit equation works very well. Thus, from the measured online power input signals, a correct online effective viscosity can be calculated although the absolute values of the online power input are not correct.

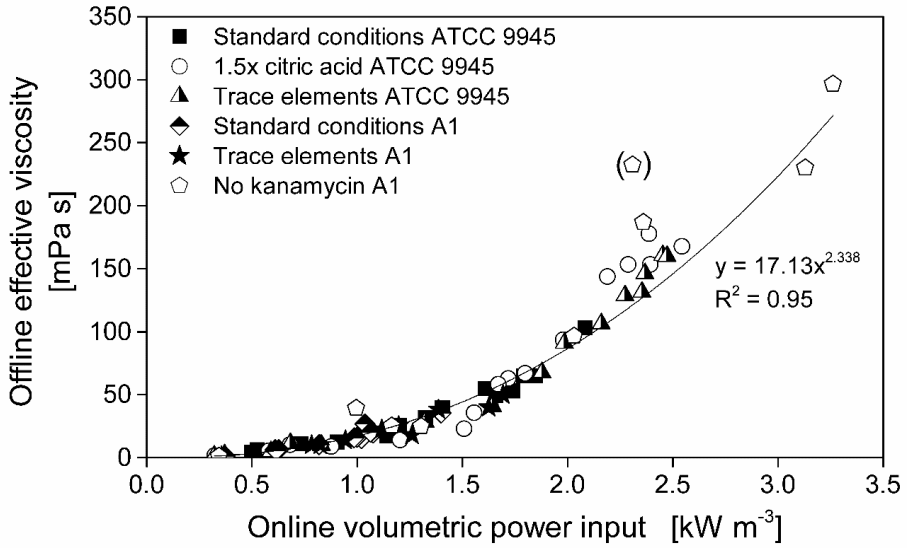

Figure 5-6 Correlation between online measured volumetric power input and offline measured effective viscosity

Initial values of the cultivations: pH = 7.4-7.6, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12/18 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4, with or w/o trace elements solution (composition see Chapter 2.4) and 50 mg L^{-1} kanamycin. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from pre-culture ($OD_{600,start}$ = 0.05).

Figure 5-7B presents the online measured power input signal and also calculated power input values determined from the offline measured effective viscosity. For this calculation, the approach and the Newton-Reynolds fit equation published by Büchs et al. (2000b) was used. The comparison between measured and calculated values clearly shows that the online power input signals measured in the experiments for this thesis are much lower than expected. Yet, despite the fact that the absolute vales are probably not correct, the online measured power input signals still correctly display the trend of the effective viscosity during the cultivations. Therefore, the power input signals described and discussed in the previous chapters can still be used to evaluate the general trend of the viscosity, reflecting the production and degradation of γ-PGA. Moreover, the signals of cultivations with different medium compositions or with different strains can be compared and evaluated regarding their absolute values, since the fit equation in Fig. 5-6 showed that the general correlation between effective viscosity and power input is still intact.

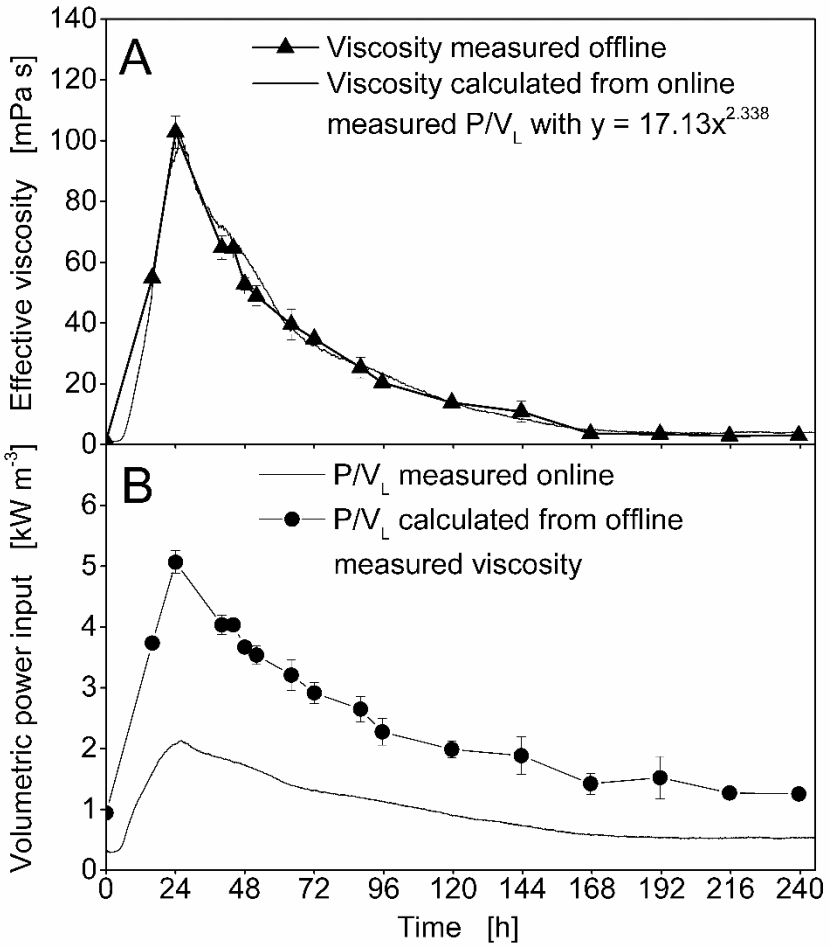

Figure 5-7 Effective viscosity and volumetric power input – measured and calculated – from a cultivation of *B. licheniformis* ATCC 9945 in standard mineral medium E for γ-PGA production

The effective viscosity determined from offline rheometer measurements and calculated from the online measured power input signal is shown in **(A)**. For the calculation the equation determined in Fig. 5-6 was used. The online measured power input signal and the volumetric power input calculated from the offline measured effective viscosity values are shown in **(B)**. Initial values of the cultivation: pH = 7.4, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from pre-culture ($OD_{600,start}$ = 0.05).

5.6 Summary

It was demonstrated that the online measurement of the volumetric power input is a valuable tool to characterize and optimize the γ-PGA production by *B. licheniformis*. The volumetric power input correlates very well with the offline determined effective viscosity and can effectively be used to follow product formation and degradation. The additional OTR measurements were useful to evaluate the general behavior and reproducibility of the respiration activity. The presented results showed that power input and viscosity depend on the molecular weight as well as the concentration of the produced γ-PGA. However, changes of molecular weight have a greater influence than changes of concentration. The observed decrease in molecular weight of γ-PGA was very likely due to the secretion of γ-PGA specific depolymerases. Especially the activity of an endo-depolymerase would have a significant impact on molecular weight and, thus, on viscosity and power input. The degradation of the produced γ-PGA started very early in the cultivation, when two of three carbon sources were still present in significant concentrations. Therefore, the trigger for the degradation of γ-PGA could not be the depletion of all carbon source in general. Also, an ammonium limitation as the trigger could be excluded, since the concentration never decreased to a limiting range.

Interestingly, it was observed that at the beginning of the γ-PGA degradation, citric acid and phosphate concentrations were relatively low. An increase of the phosphate concentration by the factor three showed no influence under standard conditions (Chapter 4.2). The increase of citric acid by 50 % led to a significant increase in effective viscosity (Chapter 4.3). The additional experiment with online power input measurement showed also a corresponding increase of the power input signal. Moreover, an increase was observed for the maximum molecular weight, but not for the maximum concentration of γ-PGA. This demonstrated that a difference in γ-PGA production, which leads for instance to a higher molecular weight, can be detected with online power input measurement. In a further experiment, trace elements were added. The addition of, e.g. cobalt, zinc, nickel, and copper, resulted in a distinctly higher effective viscosity and volumetric power input. This suggests that in standard mineral medium E, as originally published by Leonard et al. (1958), certain trace elements are missing to support unlimited growth and production.

Besides the typical γ-PGA producing strain *B. licheniformis* ATCC 9945, a second γ-PGA producing strain, *B. licheniformis* A1, was cultivated in standard mineral medium E (with kanamycin). The results illustrated how two γ-PGA producing *B. licheniformis* strains differ in their growth and product formation. It was also demonstrated that differences between strains can be detected using online measurement of volumetric power input and OTR as signals for product formation and respiration activity, respectively. In further experiments, the strain A1 was also cultivated with trace elements and one cultivation was performed under standard conditions without the antibiotic kanamycin. For these cultivations, the maximum power input and viscosity were observed much later than for the strain ATCC 9945 and also for the cultivation with kanamycin. The maxima were now observed after approximately 96 h and not after 24 h. For the cultivation of strain A1 in standard medium E without kanamycin, the highest effective viscosity and volumetric power input of all experiments were determined, which were 297 mPa s and 3.3 kW m^{-3}. In case of strain A1, the antibiotic obviously had a strong impact on γ-PGA production despite the strain's genetic resistance.

Ultimately, a fit equation was calculated from offline viscosity and online power input values from all experiments. With this fit equation it was possible to calculate an online viscosity curve using the online measured power input signal.

Chapter 6

Conclusion and outlook

Bacillus species are well known producers of homologous and heterologous proteins, antibiotics, nucleotides, bio-surfactants, biofuels, vitamins and biopolymers, such as poly(γ-glutamic acid). One major product which is manufactured in bulk quantities are proteases for the detergent industry. Almost all of these detergent proteases are produced by *Bacillus* species such as *B. licheniformis* and *B. subtilis*. The biopolymer poly(γ-glutamic acid) (γ-PGA) can either be the main and valuable product of certain *Bacillus* species, but it can also be produced as an undesired by-product in other production processes. The production of biopolymers increases the viscosity of the fermentation broth. The elevated viscosity impairs mixing and also gas/liquid mass and heat transfer in any bioreactor. If an industrial *Bacillus* production strain not only secretes the target compound, but also γ-PGA as an undesired by-product, this will massively interfere with the fermentation process itself and also with the subsequent downstream processing. As a resulting impairment or failure of fermentation and downstream processes is quite obviously economically unfavorable, it is of great interest to investigate how to prevent the formation of undesirable biopolymers and allow for undisturbed fermentation and downstream processing.

In Chapter 3, an industrial protease producing *B. licheniformis* strain (A1), which showed the described phenomenon of undesired γ-PGA formation, was presented. The influence of nitrogen source and pH value on protease and γ-PGA production was investigated in shake flask and stirred tank reactor cultivations. A simple solution was presented to avoid by-product

formation of γ-PGA without having a negative impact on protease production. The applied medium originally contained ammonium as the sole nitrogen source and glucose as the carbon source, from which *B. licheniformis* synthesized glutamate as an overflow metabolite. In the presence of this excess glutamate, γ-PGA was formed. If nitrate was added as the sole nitrogen source, no γ-PGA formation was observed. Before assimilation, nitrate must be reduced to ammonium. Using nitrate has distinctively higher energy costs than ammonium and will only be taken up and reduced in the amounts required for cell growth and maintenance. With nitrate, no excess glutamate is formed and, thus, no γ-PGA is produced.

The application of ammonium or nitrate as nitrogen sources resulted in different pH progression curves. The influence of the pH value on the undesired γ-PGA was investigated in order to distinguish between the impact of the nitrogen source and the impact of the pH value. The experiments conducted in stirred tank bioreactors demonstrated that the pH value had no influence on preventing or promoting the undesired γ-PGA formation. All of these experiments were performed under oxygen unlimited conditions. But when *B. licheniformis* was cultivated under oxygen limited conditions and with nitrate as nitrogen source, γ-PGA formation was observed. Under oxygen limited conditions, reduced cofactors like NAD(P)H accumulate due to a lack of oxygen that acts as the usual electron acceptors necessary to regenerate the reduced cofactors. The accumulated reducing agents can be oxidized by reducing nitrate to ammonium, which the cells can subsequently use in the biosynthesis of glutamate and γ-PGA. Under oxygen limited conditions, therefore, γ-PGA could be formed despite the fact that nitrate was applied as nitrogen source.

This work showed that undesired γ-PGA formation of a protease producing *Bacillus licheniformis* strain can be prevented by simply exchanging the nitrogen source in the applied mineral production medium. This change in medium composition is applicable for shake flask and stirred tank bioreactor fermentations, respectively, since the typically used pH control systems with buffer or titration did not have an influence on the undesired γ-PGA formation. Nevertheless, it has to be kept in mind, that cultivations should be conducted under oxygen unlimited conditions – otherwise γ-PGA could still be formed even with nitrate as nitrogen source.

In the future, it could be investigated whether γ-PGA really is degraded to single glutamate monomers or if the decreasing viscosity is only due to the activity of γ-PGA endodepolymerases. Therefore, experiments under oxygen limited conditions with nitrate as nitrogen source could be conducted to induce γ-PGA formation. After the time point of maximum viscosity, it could be tested whether glutamate monmers or released ammonium ions can be detected in the culture broth via enzymatic and photometric tests, respectively. Furthermore, an optimization of the product formation under the investigated fermentation conditions could be conducted to guarantee highest protease production with nitrate as nitrogen source. For instance, the nitrate concentration and the starting pH could be varied to find the optimal production conditions.

Bacillus species, especially *B. subtilis* and *B. licheniformis*, are also used to produce γ-PGA as the main valuable product. A typical *B. licheniformis* production strain (ATCC 9945) for γ-PGA was investigated. Its growth and γ-PGA production was characterized under standard conditions adapted from the literature. Furthermore, the impact of different medium compositions on γ-PGA formation was investigated and the influence of different oxygen transfer capacities was studied by varying the filling volume of the shake flasks. The results of these experiments were described and discussed in detail in Chapter 4.

Independently of the medium composition, a deviation of the cultures' respiration and metabolic activities, respectively, was observed. This divergence resulted in the early termination of respiration activity of some of the cultures, which coincided with low final pH values in the respective flasks. As a reason for the deviation of the cultures the formation of subpopulations due to spore formation was suggested. The initiation of spore formation and sporulation itself is a complex process, involving numerous regulator proteins, enzymes, and also toxins for killing non-sporulating cells (González-Pastor, 2011; Piggot and Hilbert, 2004). For instance under nutrient limited conditions, the master regulator of sporulation in *Bacillus* (Spo0A) becomes active, but only in part of the cell population. Cells at the onset of sporulation secrete extracellular toxins – killing factors – that lead to lysis of non-sporulating cells. The sporulating cells are immune to their own killing factors, two proteins, of which at least one can be purified from medium containing sporulating cells. The lysis of non-sporulating cells releases nutrients which the sporulating cells can feed on. Until a certain point in the sporulation process the cells are not fully committed to spore formation and the

process can be arrested and cells can resume growth and cell division. The sporulation process requires a lot of energy and several hours to be completed. The killing of non-sporulating cells and the concomitant release of nutrients allow for the not fully committed sporulating cells to arrest spore formation until the new nutrients are exhausted. Starvation will again divide the cells into Spo0A-active and -inactive populations and further rounds of killing will take place until most of the remaining population has fully transformed into spores or until nutrients are available again in the medium. These cycles of killing are responsible for a significant delay of sporulation. Sustaining a population of sporulating and vegetative cells as long as possible helps *Bacillus* communities to fast and easily resume full growth and cell division if the environmental conditions improve (González-Pastor, 2011).

To exactly determine the triggers for the different development of the cultures which results in the significant divergence of the respiration activities and is probably caused by the initiation of sporulation would be a time and work intensive process. Therefore, the sporulation mechanism of the strain *B. licheniformis* ATCC 9945 should be completely inactivated by genetic engineering. This is usually performed for industrial *Bacillus* production strains, as it was done for strain A1, by deleting or inactivating the gene for the transcription factor Spo0A (Tännler et al., 2008). Thereby, it could be proven whether spore formation is the trigger for the divergence of the cultures and, at once, the reproducibility of the cultivations could be greatly improved.

The maximum effective viscosity was achieved after 48 h and, hence, before the earliest diverging of the cultures, which was observed after 60 h. It was assumed that the γ-PGA production phase is similar for all cultures and the deviation does not interfere with the production process. The maximum viscosity coincided with the depletion of the carbon source citric acid and a limitation of phosphate. The decrease in viscosity after its maximum indicated that an unidentified factor triggered the enzymatic degradation of γ-PGA. Therefore, the influence of higher initial phosphate and citric acid concentrations on γ-PGA production was studied to determine whether the depletion of one of these compounds played a role in the enzymatic degradation of γ-PGA. A non-limiting phosphate concentration did not influence the γ-PGA production. However, the increase of the initial citric acid concentration by 50 % led to a doubling of the maximum viscosity and thereby demonstrated the significant influence of citric acid on γ-PGA formation. The higher citric acid concentration resulted in

considerably higher ammonium consumption, so that an ammonium limitation during cultivation could not be fully excluded. Following this, the influence of a higher citric acid concentration combined with a higher ammonium concentration or a higher phosphate concentration was investigated. The results reconfirmed the impact of an increased citric acid concentration on the γ-PGA production process. The parallel increase of the phosphate concentration further increased the maximum viscosity. In contrast, the higher ammonium concentration prevented a limitation but did not result in a higher maximum viscosity compared with additional citric acid only. The higher consumption of ammonium led to a strong decrease of the pH value, down to values no longer optimal for the growth of *B. licheniformis*, which resulted in an early termination of respiration activity of all cultures.

For future investigations, the influence of even higher citric acid concentrations on γ-PGA production should be investigated. At the same time, glutamic acid and glycerol concentrations could be varied, to determine if the high initial concentrations of these carbon sources are essential for effective product formation or not. For instance, at the time point of maximum viscosity, high residual concentrations of glycerol are still present in the medium. Therefore, the initial glycerol concentration can possibly be decreased. If higher citric acid concentrations are applied, ammonium (= nitrogen) limitations have to be avoided. Since the increase of the ammonium concentration prevented a nitrogen limitation but simultaneously led to a too low pH value, the nitrogen limitation could also be avoided by supplying a different nitrogen source, for instance nitrate. The consumption of nitrate would increase the pH value and thereby prevent the strong decrease of the pH value to non-physiological values. Changing part of the standard ammonium concentration to nitrate could already be beneficial for cultivations under standard conditions. For all cultivations, a deviation of the respiration activities accompanied by an early termination of respiration activity for some or all of the parallel cultures was observed. This phenomenon coincided with very low pH values, from which some of the cultures could not recover. The application of nitrate together with ammonium as nitrogen source could probably efficiently prevent that the pH value decreases too much and could therefore increase the reproducibility of the parallel cultures. The reduced cofactors, e.g. NADH, which are necessary for the reduction of nitrate to ammonium are easily provided by the cells since under oxygen limited conditions usually more reduced cofactors in their reduced form are accumulated than under oxygen unlimited conditions, for instance. The

application of nitrate should therefore not hamper the production of γ-PGA (compare Chapter 3.3).

The variation of the filling volume demonstrated a strong impact of the maximum oxygen transfer capacity on respiration activity and γ-PGA production. On the one hand, high maximum oxygen transfer capacities were beneficial for the reproducibility of the cultures and promoted a long period of respiration activity. On the other hand, cultures with low maximum oxygen transfer capacities resulting in long periods of oxygen limitation often showed an early termination of respiration activity. With decreasing oxygen transfer capacity, an increase of the effective viscosity was observed. The highest viscosity was obtained with a maximum oxygen transfer capacity of 20 mmol L^{-1} h^{-1}. For the lower maximum oxygen transfer capacity of the standard filling volume, a significantly lower maximum effective viscosity was determined. It was concluded that a maximum oxygen transfer capacity resulting in a certain level of oxygen limitation is beneficial for γ-PGA production. However, if the maximum oxygen transfer capacity is too low, this has a negative impact on respiration activity and product formation. Therefore, the optimal level of oxygen limitation should be determined by further investigations with different filling volumes.

It was shown that the trigger for the start of the enzymatic degradation is apparently neither the phosphate limitation nor the depletion of citric acid. The depletion or a lack of another nutrient such as a trace element could also initiate the degradation. The experiment with different filling volumes revealed that a second substrate limitation occurred before phosphate or citric acid were depleted. Therefore, in following experiments, also a supplementation with trace elements was investigated.

Chapter 5 described the application of power input measurement for shake flask cultivations to follow γ-PGA formation and degradation. For these investigations, the *B. licheniformis* γ-PGA production strain (ATCC 9945) and the protease producing *B. licheniformis* strain (A1) with the undesired γ-PGA formation were analyzed and compared regarding their growth and production behavior. It was demonstrated that online measurement techniques for the determination of volumetric power input and OTR are valuable tools to characterize and optimize γ-PGA production by *B. licheniformis*. The volumetric power input correlated very well with the offline determined effective viscosity and can effectively be used to follow

product formation and degradation. OTR measurements are useful to evaluate the general behavior and reproducibility of respiration activity. Power input and viscosity depended on molecular weight and concentration of the produced γ-PGA, but changes of the molecular weight had a higher influence than changes of the concentration. The observed decrease in molecular weight of γ-PGA is probably caused by the secretion of γ-PGA specific depolymerases.

Chapter 4.3 had successfully demonstrated the influence of a higher citric acid concentration on γ-PGA formation. The increase of citric acid by 50 % led to a significant increase in effective viscosity. The experiment with additional online power input measurement showed also a corresponding increase of the power input signal (Chapter 5.2). Moreover, an increase was observed for the maximum molecular weight, but not for the maximum concentration of γ-PGA. This demonstrated that a difference in γ-PGA production which resulted in a higher molecular weight can be detected with online power input measurement. In a further experiment, trace elements were added. The addition resulted in a distinctly higher effective viscosity and volumetric power input. This suggests that in standard mineral medium E, as originally published by Leonard et al. (1958), certain trace elements are missing to support unlimited growth and production. The maximum viscosity was still obtained at the same point of time as in other experiments and also showed a significant decrease after its maximum. The supplemented trace elements, just as higher phosphate and citric acid concentration, could also not prevent or delay the enzymatic degradation of the γ-PGA.

Besides the typical γ-PGA producing strain *B. licheniformis* ATCC 9945, a second γ-PGA producing strain, *B. licheniformis* A1, was cultivated in standard mineral medium E (with kanamycin). The results illustrated that differences between strains can be detected using online measurement of volumetric power input and OTR as signals for product formation and respiration activity, respectively. The strain A1 was also cultivated with trace elements and under standard conditions without the antibiotic kanamycin. For these cultivations, the maximum power input and viscosity were obtained at a later time point than for the strain ATCC 9945 and also for the cultivation with kanamycin. For the cultivation of strain A1 in standard medium E without kanamycin the highest effective viscosity and volumetric power input of all experiments was determined. Finally, a fit equation was calculated from offline

viscosity and online power input values from all experiments. With this fit equation it was possible to calculate an online viscosity curve using the online measured power input signal.

In this work, the production of the industrially relevant biopolymer poly(γ-glutamic acid) by *Bacillus licheniformis* was thoroughly characterized. Different factors influencing the product formation were identified. Moreover, online monitoring of γ-PGA formation and degradation by measuring the volumetric power input was successfully applied.

Appendix

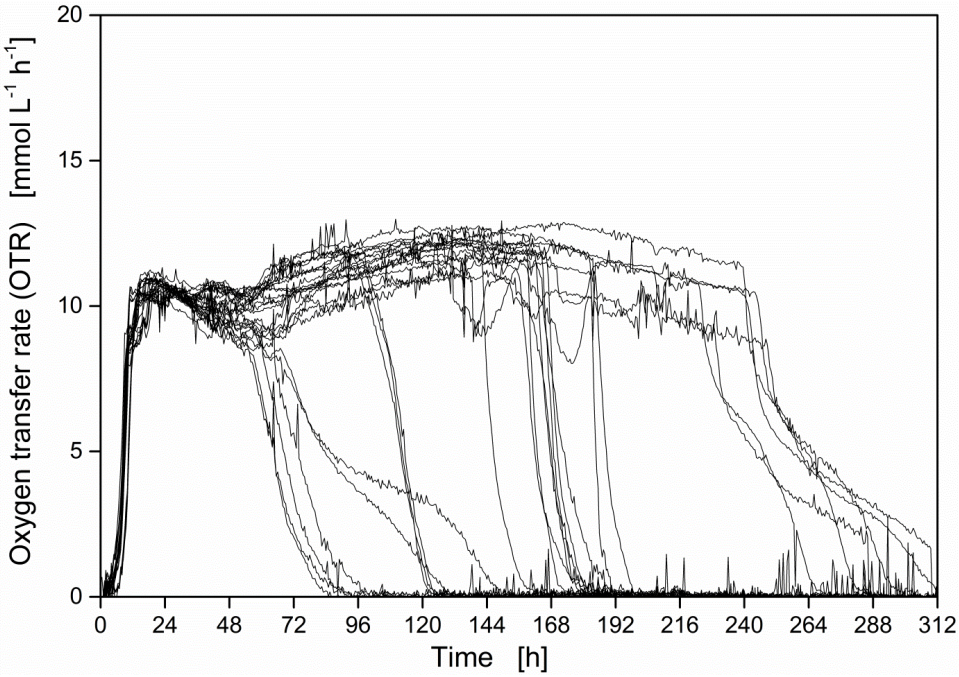

Figure 7-1 Oxygen transfer rates of *B. licheniformis* ATCC 9945 cultivations in standard mineral medium E

Oxygen transfer rates (OTR) of 34 RAMOS shake flasks from ten individual experiments. Initial values: pH = 7.4, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from glycerol stock (0.4 % v/v).

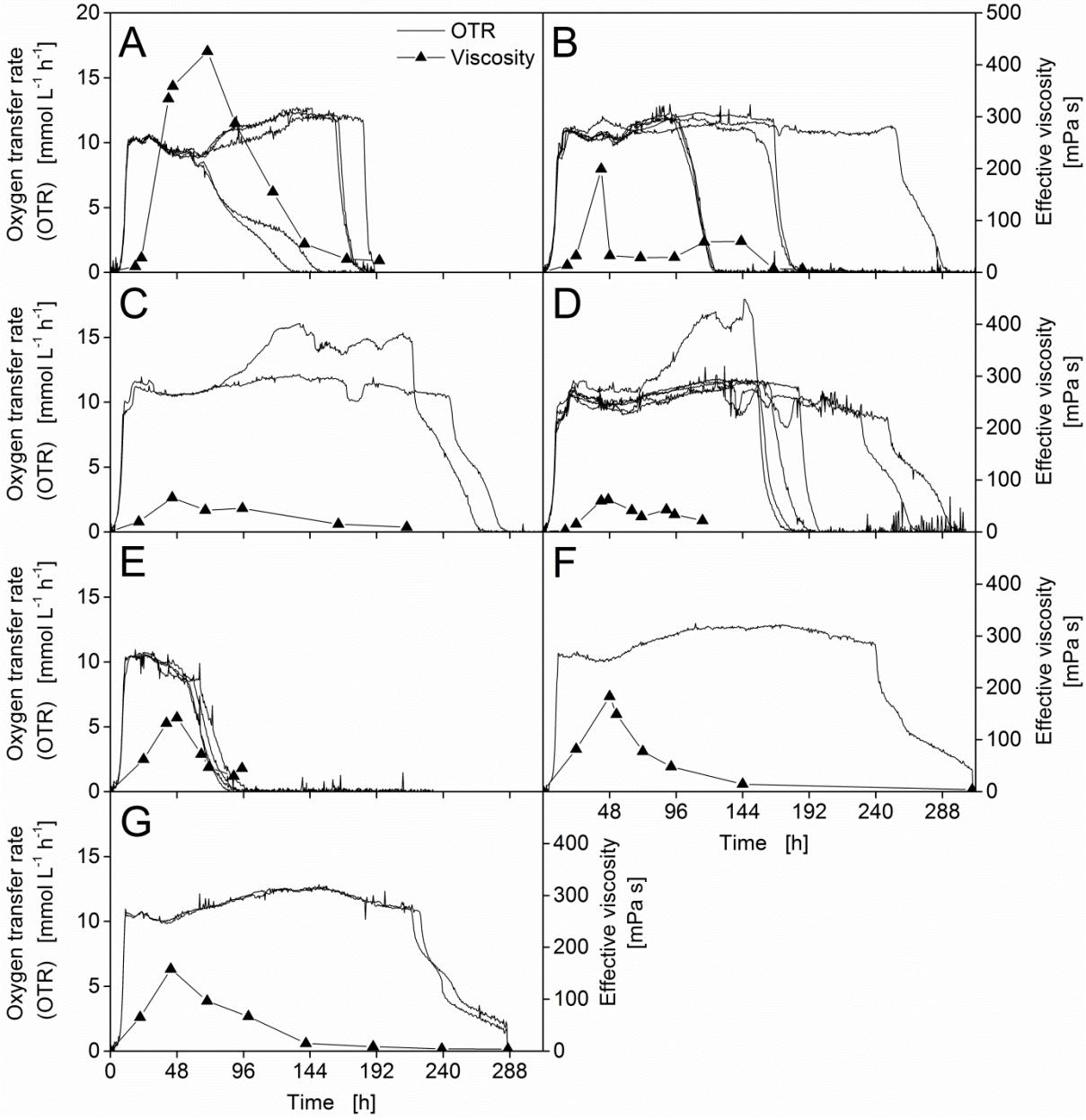

Figure 7-2 Oxygen transfer rates and effective viscosity of *B. licheniformis* ATCC 9945 cultivations in standard mineral medium E

Results of seven individual experiments **(A-G)**. Experiment N°3, May 2013 **(A)**. Experiment N°4, August 2013 **(B)**. Experiment N°5, September 2013 **(C)**. Experiment N°9, December 2013 **(D)**. Experiment N°13, January 2014 **(E)**. Experiment N°15, April 2014 **(F)**. Experiment N°16, June 2014 **(G)**. Initial values: pH = 7.4, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from glycerol stock (0.4 % v/v).

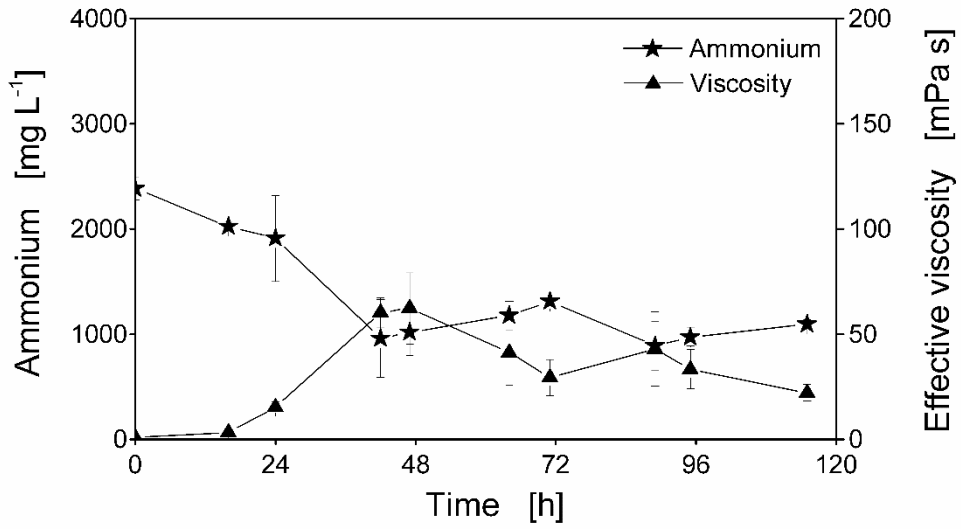

Figure 7-3 Trends of ammonium concentration and effective viscosity of *B. licheniformis* ATCC 9945 under standard conditions

Initial values: pH = 7.6, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from glycerol stock (0.4 % v/v).

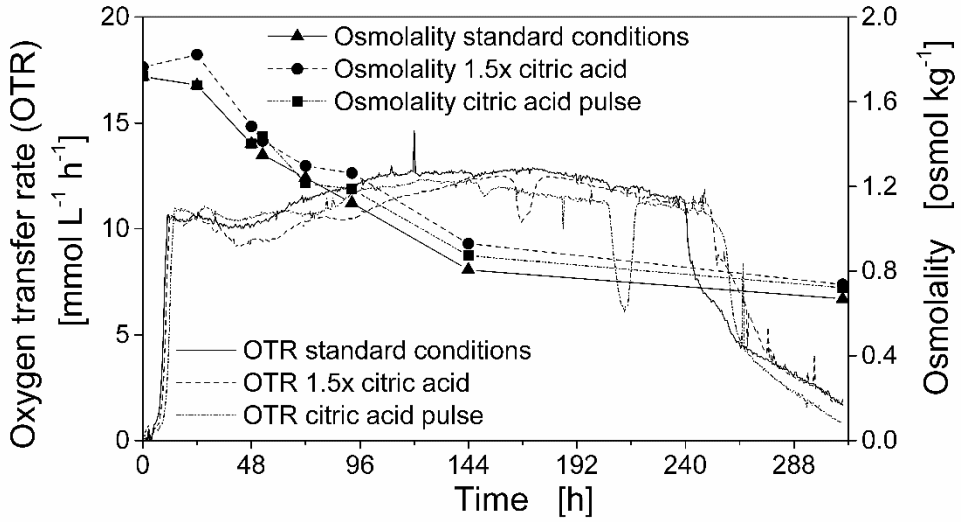

Figure 7-4 Trends of oxygen transfer rates and osmolality of *B. licheniformis* ATCC 9945 under standard conditions, with 1.5x citric acid or with a citric acid pulse (after 48 h)

Initial values: pH = 7.5-7.6, 80 g L^{-1} glycerol, 20 g L^{-1} glutamic acid, 12 or 18 or 12 plus 6 g L^{-1} citric acid, 7 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4. Cultivation conditions: T = 37 °C, 500 mL shake flasks, filling volume 100 mL, shaking frequency 250 rpm, shaking diameter 50 mm, inoculation from glycerol stock (0.4 % v/v).

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