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FERMENTATION, CELL CULTURE AND BIOENGINEERING

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

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Abstract *Bacillus* spp. are used for the production of industrial enzymes but are also known to be capable of producing biopolymers such as poly(γ-glutamic acid). Biopolymers increase the viscosity of the fermentation broth, thereby impairing mixing, gas/liquid mass and heat transfer in any bioreactor system. Undesired biopolymer formation has a significant impact on the fermentation and downstream processing performance. This study shows how undesirable poly(γ-glutamic acid) formation of an industrial protease producing *Bacillus licheniformis* strain was prevented by switching the nitrogen source from ammonium to nitrate. The viscosity was reduced from 32 to 2.5 mPa s. A constant or changing pH value did not influence the poly(γ-glutamic acid) production. Protease production was not affected: protease activities of 38 and 46 U mL^{-1} were obtained for ammonium and nitrate, respectively. With the presented results, protease production with industrial *Bacillus* strains is now possible without the negative impact on fermentation and downstream processing by undesired poly(γ-glutamic acid) formation.

Keywords Protease and amylase production · *Bacillus licheniformis* · Poly(γ-glutamic acid) · Ammonium · Nitrate

Abbreviations

γ-PGA	Poly(γ-glutamic acid)
DOT	Dissolved oxygen tension (%)
OD_{600}	Optical density at 600 nm (−)
OTR	Oxygen transfer rate (mmol L^{-1} h^{-1})

Introduction

Bacillus spp. are well-known producers of homologous and heterologous proteins, antibiotics, nucleotides, biosurfactants, biofuels, and also biopolymers [17, 22, 30]. One major product which is manufactured in bulk quantities is proteases for the detergent industry [25]. This industry accounts for more than one-third of the industrial enzyme market and almost all detergent proteases are produced by *Bacillus* spp. In 2012, the market for industrial enzymes was estimated to be 3.9 billion US$ [7].

Another product of many *Bacillus* spp. is poly(γ-glutamic acid) (γ-PGA): an anionic, water-soluble, and biodegradable biopolymer. γ-PGA consists of up to 10,000 D- and/or L-glutamic acid monomers which are linked by γ-peptide bonds. This property makes γ-PGA stable against proteases, but it can either be degraded chemically or enzymatically by specific depolymerases, often also expressed under specific conditions by the producing organisms. Microbial produced γ-PGA by *Bacillus* spp. has generally

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a high-molecular weight in the range of 10–1000 kDa and a broad polydispersity [6, 10]. *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.

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 [20]. Previous studies showed that the broth's viscosity impairs mixing and also gas/liquid mass and heat transfer [9, 13]. 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 [9]. This "out-of-phase" condition leads to a significantly reduced power input into the liquid and, thus, to strongly reduced mixing and mass transfers. Accordingly, its occurrence is very unfavorable for fermentations in shake flasks and should be avoided at all times [14]. Furthermore, the production of a biopolymer with pseudo-plastic properties also impacts stirred tank bioreactor fermentations. Giese et al. [14] 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. To sum it up, 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 undesired by-products in form of biopolymers such as γ-PGA, this will massively interfere with the fermentation process itself and also with the subsequent downstream processing. It is clearly comprehensible that such impaired or even failed fermentations or downstream processes are time-wasting and economically unfavorable. Although this phenomenon is frequently noticed [11, 15, 18], there are many studies about improving γ-PGA production, but only little work dealing with the prevention of undesired γ-PGA formation [19, 32]. Probably, in some cases, impaired or failed fermentations or down-stream processing are encountered, without identifying the reason for these problems. Therefore, it is of great interest to investigate how to prevent the formation of undesirable biopolymers to allow for undisturbed fermentations and downstream processing.

This study demonstrates the phenomenon of undesired γ-PGA production of an industrial protease producing *Bacillus licheniformis* strain and presents 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 this study's 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 this study. Therefore, the simple means of viscosity measurements with a rheometer were chosen to easily follow γ-PGA production during fermentations compared to more complicated and time-consuming analytical techniques like gel permeation chromatography.

Materials and methods

Microbial strain and media

The protease producing *Bacillus licheniformis* strain was kindly provided by Henkel AG & Co. KGaA (Düsseldorf, Germany). 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.

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 7.0–7.2. Prior to cultivation, kanamycin sulfate was added to the autoclaved pre-culture medium from a sterile filtered stock solution to give a final concentration of 50 mg L^{-1}.

The main culture medium V3 [32] for shake flask cultivations and stirred tank bioreactor cultivations with a pH-buffer system contained per liter: 20 g glucose, 1.01 g $MgSO_4$ $7H_2O$, 0.026 g $CaCl_2$ $2H_2O$, 0.05 g $MnCl_2$ $4H_2O$, 15 g $(NH_4)_2SO_4$, 5 mL trace elements stock solution (preparation see below), 0.05 g $FeSO_4$ $7H_2O$, 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 1000× 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 200× stock

solution, sterile filtered and used for medium preparation. The $FeSO_4$ $7H_2O$ 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 $(NH_4)_2SO_4$, $MgSO_4$ H_2O, $CaCl_2$ H_2O, and $MnCl_2$ $4H_2O$ 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 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), $FeSO_4$ $7H_2O$ stock, MOPS acid with a pH value of 8, K_2HPO_4, kanamycin sulfate. Before adding K_2HPO_4, the pH was checked and, if necessary, re-adjusted with 5 M NaOH to pH 8 for experiments with MOPS buffer or to pH 7 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.

Cultivation conditions and determination of oxygen transfer rate

A two-stage pre-culture was used to inoculate main cultures. The first pre-culture was inoculated from a glycerol stock, which was stored 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, 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 with 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. On-line 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) [2, 3]. Commercial versions are available from Kuhner AG, Birsfelden, Switzerland or HiTech Zang, Herzogenrath, Germany.

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 offline 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 shake flask and stirred tank reactor experiments, the O_2 consumption of *Bacillus licheniformis* was determined via the OTR. For shake flask cultivations, the OTR was calculated from the measurement of the oxygen partial pressure in the head space of the RAMOS shake flasks using the RAMOS device [2, 3]. The equation for OTR calculation is given by Eq. 1, with the moles of oxygen n_{O_2} (mol), the liquid filling volume of the RAMOS shake flask V_{fl} (L), the time t (h), the difference of oxygen partial pressure Δp_{O_2} (bar), the time of the measuring phase Δt (h), the gas volume of the RAMOS shake flask V_g (L), the gas constant $R = 8.314$ (J mol^{-1} K^{-1}), and the temperature T (K):

$$\mathrm{OTR} = \frac{n_{O_2}}{V_{fl} \cdot t} = \frac{\Delta p_{O_2}}{\Delta t} \cdot \frac{V_g}{R \cdot T \cdot V_{fl}}. \quad (1)$$

For main cultures in stirred tank bioreactors, a 3 L BIOSTAT Bplus (Sartorius Stedim Biotech GmbH, Göttingen, Germany) was used, equipped with temperature (Pt100), dissolved oxygen tension (DOT) and pH probes, 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. The cultivation temperature for all experiments 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 adjusted in the range of 100–2000 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 reactor cultivations, O_2 and CO_2 concentrations were measured using an exhaust gas analyzer (DASGIP GA4, Eppendorf, Wesseling-Berzdorf, Germany). The OTR was calculated from Eq. 2 [24], with the

aeration rate Q_g at standard conditions (L min^{-1}), the molar volume of an ideal gas at standard conditions $V_m = 22.414$ (L mol^{-1}), the stirred tank reactor filling volume V_l (L), the oxygen mole fraction of the inlet gas $y_{O_2,in}$ (−), the oxygen mole fraction of the exhaust gas $y_{O_2,out}$ (−), the carbon dioxide mole fraction of the inlet gas $y_{CO_2,in}$ (−), and the carbon dioxide fraction of the exhaust gas $y_{CO_2,out}$ (−):

$$\mathrm{OTR} = \frac{60 \cdot Q_g}{V_m \cdot V_l} \cdot \left[y_{O_2,\,in} - \left(\frac{1 - y_{O_2,in} - y_{CO_2,in}}{1 - y_{O_2,out} - y_{CO_2,out}} \right) \cdot y_{O_2,out} \right]. \quad (2)$$

Under quasi steady-state conditions, it is justified to assume that the OTR is equal to the oxygen uptake rate (OUR) of the microorganisms.

Biomass analysis

Biomass was determined by optical density measurement at 600 nm (Genesys 20 Visible Spectrophotometer, Thermo Scientific, Waltham, USA). Samples were diluted appropriately (between 1:2 and 1:50) with 0.9 % NaCl solution. Cell dry weight (CDW) was also determined but was found to be strongly influenced by γ-PGA formation. Therefore, only data for optical density measurement are shown. For approximate cell dry weight quantification, a correlation between optical density and cell dry weight was established using the data from the experiments with nitrate as nitrogen source and under glucose and oxygen unlimited conditions. The following correlation equation was obtained:

$$\mathrm{CDW}\ [\mathrm{g\,L^{-1}}] = 0.676 \cdot \mathrm{OD}_{600} + 1.274 \quad (3)$$

Viscosity measurements

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 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 was used for viscosity measurements at 37 °C. As γ-PGA solutions exhibit pseudo-plastic properties, viscosity values for a distinct shear rate of $\gamma = 300$ s^{-1} were used to compare the viscosities of different experiments with each other. Viscosity measurements were chosen as a simple, general method to follow γ-PGA formation and degradation during the cultivation.

Protease activity measurements

The protease activity was measured according to the method developed by DelMar et al. [12]. 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 (1:20–1:1280) 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 the extinction coefficient of pNA at 405 nm (8.48 cm^2 µmol^{-1}). Moreover, the maximum protease formation rate was calculated using the data of the measured protease activity and the established OD-CDW correlation (Eq. 3).

Glucose and overflow metabolites

Concentrations of glucose and overflow metabolites (acetate, acetoin, 2,3-butanediol) were determined by HPLC (Ultimate 3000, Dionex, USA) equipped with an Organic Acid-Resin-Column (250 × 8 mm, CS-Chromatographie Service GmbH, Langerwehe, Germany) and an Organic Acid-Resin-Precolumn (40 × 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).

Results and discussion

Influence of ammonium sulfate on viscosity build-up in shake flask fermentations

This study investigated the influence of ammonium and nitrate as nitrogen sources on viscosity build-up of a

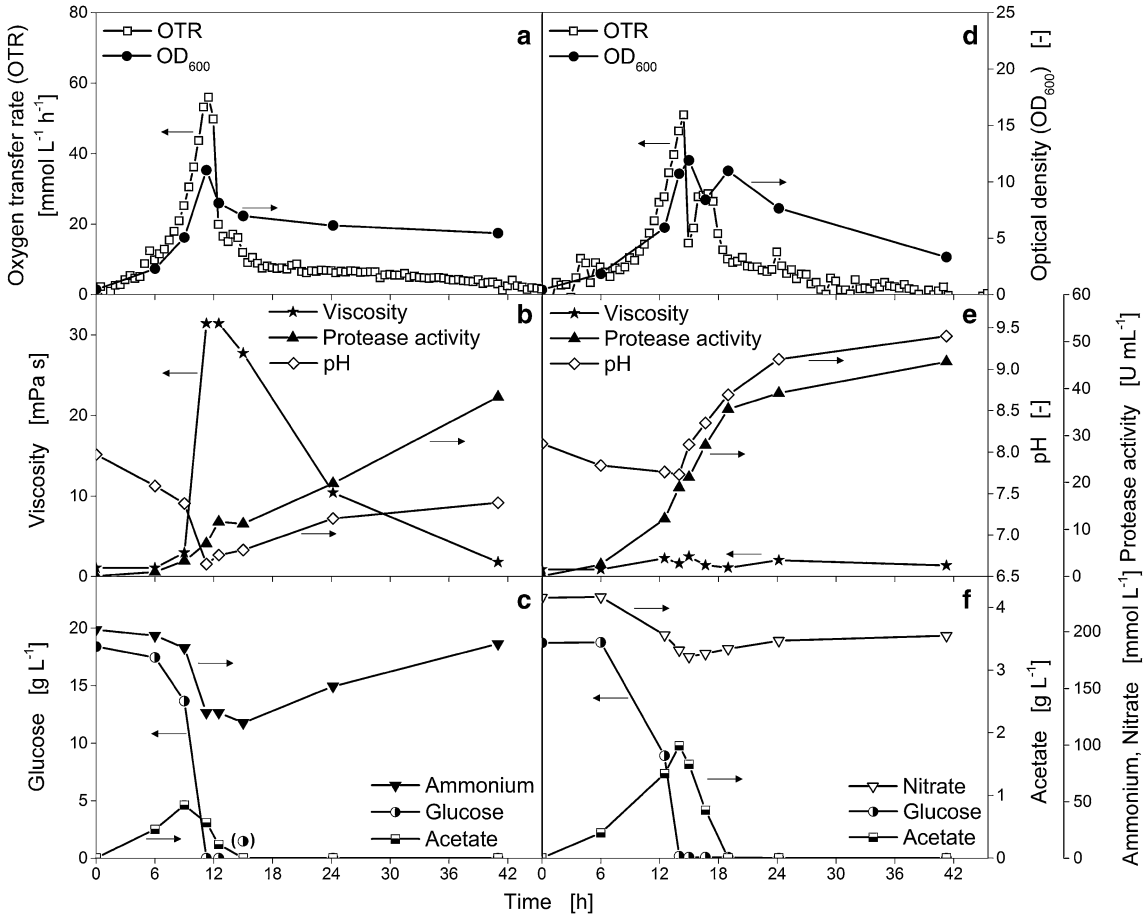

Fig. 1 Impact of nitrogen source on viscosity build-up due to poly(γ-glutamic acid) formation during fermentation in a mineral medium: comparison of *Bacillus licheniformis* cultivations in shake flasks with medium containing ammonium (**a**–**c**) or nitrate (**d**–**f**) as nitrogen source. Oxygen 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: $OD_{600} = 0.4$, $pH_0 = 8.0$, 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

protease producing *B. licheniformis* strain in shake flask fermentations. Figure 1 displays the results of these shake flask fermentations. In the mineral medium used for protease production, ammonium sulfate is applied as nitrogen source (Fig. 1a, b, c). Figure 1a displays 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 the *B. licheniformis* strain. The OTR reached a maximum of 56 mmol L^{-1} h^{-1} before declining sharply, thereby notifying the depletion of glucose (Fig. 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. 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. 1c). The time point of the minimum pH value correlated with the maximum OTR. 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. 1b). Various *Bacillus* species are known to produce the biopolymer poly(γ-glutamic acid) [6, 10]. Therefore, it was suspected that the viscosity elevating by-product was γ-PGA, which is undesirably formed by the protease producing *B. licheniformis* strain used in this study.

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 *Bacillus subtilis* strains have been identified, one acting in an endopeptidase-like fashion, cleaving the polymer into smaller oligomers [5]. These oligomers are further cleaved by an exopeptidase into single glutamate monomers [1]. 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 a final concentration of 190 mmol L^{-1} at the end of the fermentation.

Protease production was rather low during the exponential growth phase up to 12 h (Fig. 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.

Influence of potassium nitrate on viscosity build-up in shake flask fermentations

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 Fig. 1d, e, f. As for the reference cultivation (Fig. 1a, b, 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. 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. 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. 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.

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

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 and compared with ammonium sulfate and potassium nitrate. OTR curves and results of viscosity measurements are depicted in Fig. 2. The OTRs of all four approaches show an exponential increase in the first 13 h of cultivation (Fig. 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 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 noticeable from the data of the RAMOS online measuring technique. It indicates the time point of the maximum viscosity (compare Fig. 1a, b). For both cultures with ammonium as nitrogen source, viscosities of more than 25 mPa s

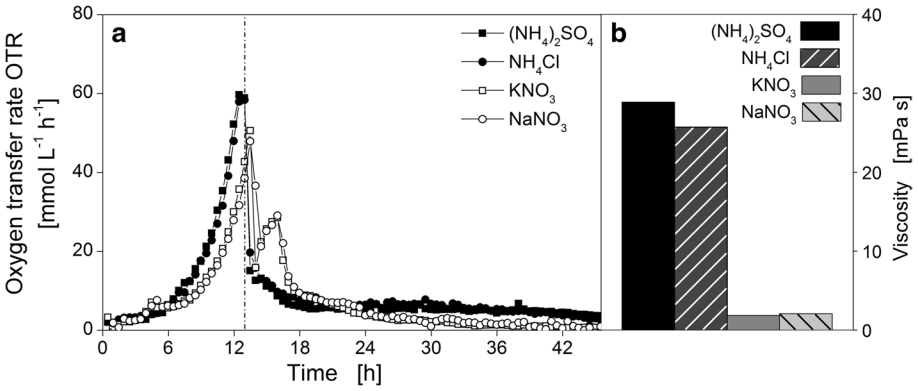

Fig. 2 Impact of two ammonium and two nitrate salts on viscosity build-up due to poly(γ-glutamic acid) formation during fermentation in a mineral medium: comparison of *Bacillus licheniformis* cultivations in shake flasks with media containing ammonium sulfate, ammonium chloride, potassium nitrate, or sodium nitrate as nitrogen source. Oxygen transfer rates (OTRs) 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: $OD_0 = 0.4$, $pH_0 = 8.0$, 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

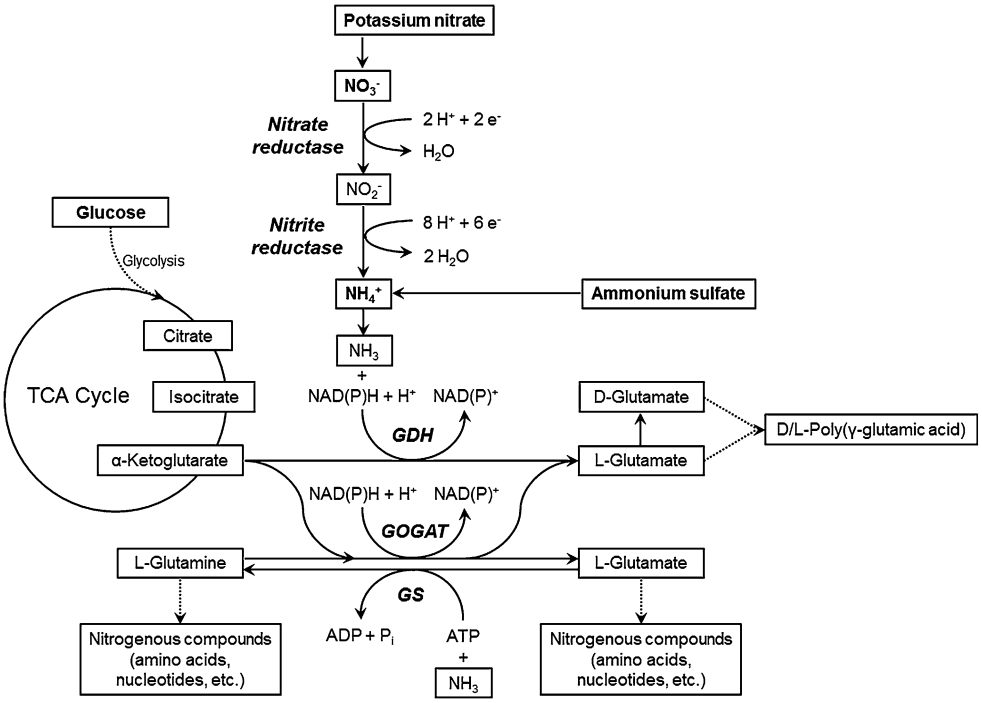

Fig. 3 Pathways of ammonium and nitrate utilization as nitrogen sources in *Bacillus licheniformis*. *GDH* glutamate dehydrogenase, *GS* glutamine synthetase, *GOGAT* glutamate synthase

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.

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. *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) [8, 31]. At high ammonium concentrations, the levels of GDH and GOGAT are high, respectively [8]. 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 [26]. 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 [31].

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). 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 [27, 28]. For their reductase activity, both enzymes require several mol NAD(P)H per mol nitrate as electron and proton donators [29]. 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.

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

In shake flask cultivations with a buffer system for pH control, 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 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 and 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 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: Fig. 4a–c displays a fermentation with MOPS buffer for pH control and Fig. 4d–f shows 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 buffer for pH control also had an initial pH value of 8.0, just like the reference in shake flasks (compare Fig. 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 are comparable. 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 cell 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 production were partially removed from the medium by the precipitation. 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. 4b, 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. The final protease activity was 31 U mL^{-1} (Fig. 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. 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 detected, 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. 4f). In this fermentation, a protease activity of only 18 U mL^{-1} was reached, which are roughly 50 % compared to the respective reference cultivation in shake flasks (Fig. 1a, b, 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 the shake flask cultivation system with nitrate as nitrogen source, the same kind of scale-up from 250 mL shake flasks to a 3 L stirred tank bioreactor was conducted. Figure 5a–c displays 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 Fig. 4a, b, c, a

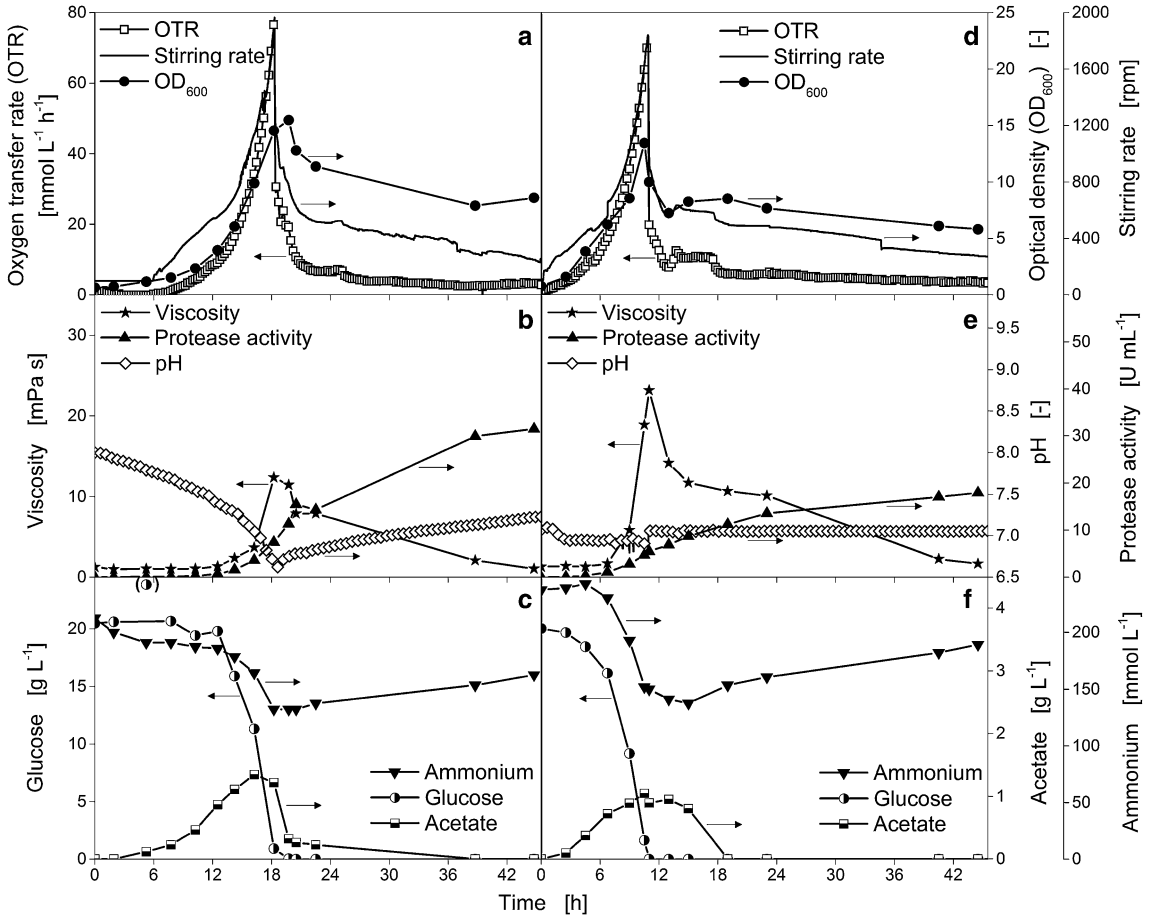

Fig. 4 Impact of pH control on viscosity build-up due to poly(γ-glutamic acid) formation during fermentation in a mineral medium containing ammonium as nitrogen source: comparison of *Bacillus licheniformis* cultivations in 3 L stirred tank bioreactors. Cultivations were performed using either buffer (**a**–**c**) or titration (**d**–**f**) for pH control. 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 and 0.17 g L^{-1} were detected after 11 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, $V_L = 1700$ mL

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. 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. 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.

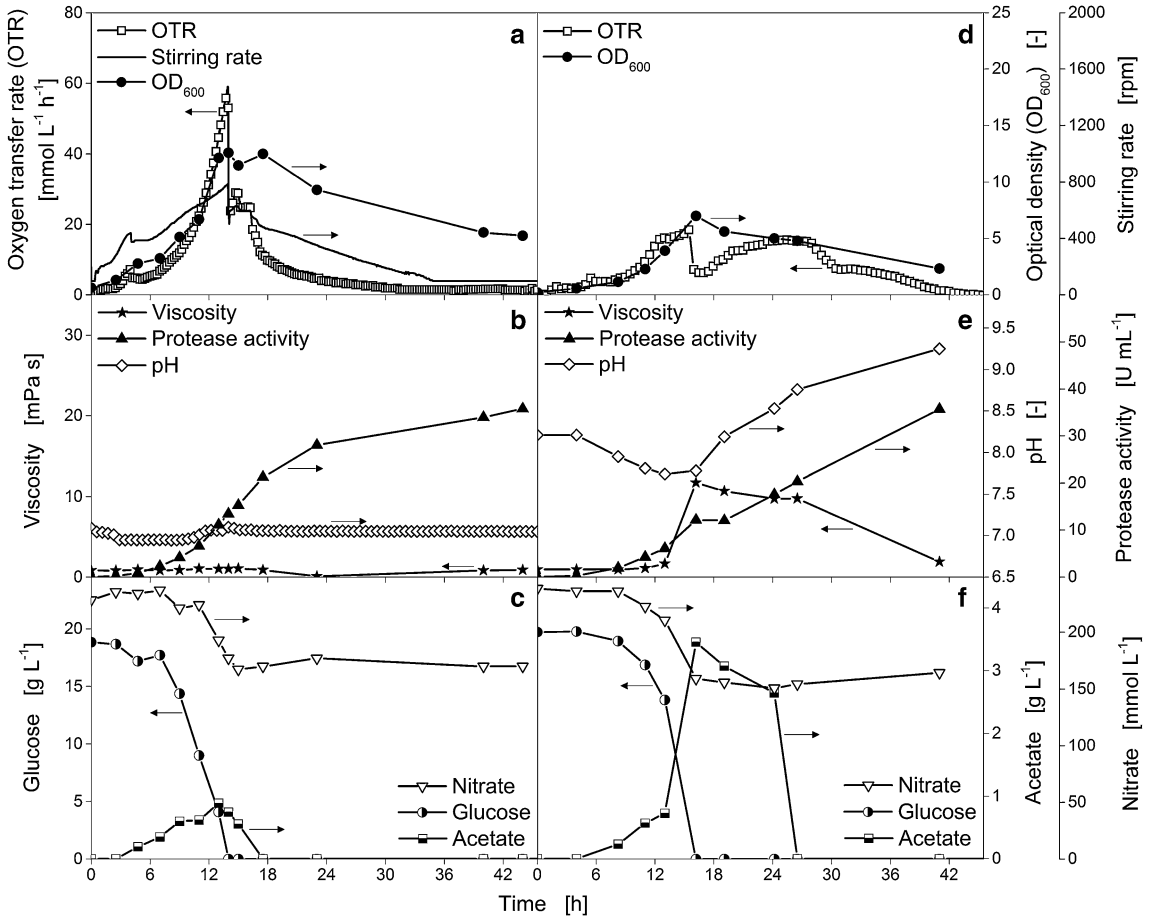

Fig. 5 Impact of pH control and oxygen limitation on viscosity build-up due to poly(γ-glutamic acid) formation during fermentation in a mineral medium containing nitrate as nitrogen source: cultivation of *Bacillus licheniformis* in a 3 L stirred tank bioreactor with titration for pH control (**a**–**c**). In the stirred tank reactor, 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 (**a**–**c**). Cultivation of *Bacillus licheniformis* in shake flasks with medium containing 0.2 M MOPS buffer (**d**–**f**). In the shake flask experiment, the filling volume was elevated and the shaking frequency reduced to impose oxygen limitation (**d**–**f**). 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 nitrate concentrations are shown in (**c**) and (**f**). In the pH-controlled fermentation, 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 (**a**–**c**). In the shake flask experiment, neither acetoin nor 2,3-butanediol was detected in any of the samples (**d**–**f**). Initial values: $OD_{600} = 0.4$, 20 g L^{-1} glucose, 22.95 g L^{-1} KNO_3. Cultivation conditions: $T = 37$ °C; $V_{L,\ stirred\ tank} = 1700$ mL (**a**–**c**); 250 mL shake flasks, filling volume 25 mL, shaking frequency 200 rpm, shaking diameter 50 mm (**d**–**f**)

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

If nitrate itself 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, the use of nitrate as nitrogen source is far more energy consuming than the use of ammonium. The cells will, therefore, only take up and reduce nitrate 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 [4, 16]. 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 are in excess and 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. [23] 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 extra 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. 1. The results of this experiment are depicted in Fig. 5d, e, f. Due to higher filling volume and lower shaking frequency, the maximum OTR reached was only 19 mmol L^{-1} h^{-1} (Fig. 5d). 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. 5f). Because of the oxygen limitation, more acetate was formed than in all 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 fermentation to a minimum of 7.7 (Fig. 5e). 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. 5f). 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. 5e). This maximum coincides with the depletion of glucose and the maximum acetate concentration. The OD_{600} increased exponentially to a maximum of 7 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. 5e). 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. 1b). In the nitrate experiments, half or even more of the protease was produced in the first growth phase on glucose (compare Fig. 1e). The exemption is the experiment with nitrate under oxygen-unlimited conditions, where more than half of the protease was produced in the second growth phase on γ-PGA and acetate. It can be concluded that with ammonium, protease production is very even throughout the whole fermentation. With nitrate, the protease formation rate is usually highest during the growth on glucose.

The increase of viscosity in the experiment depicted in Fig. 5e 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. [32] 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 study clearly fortify the findings of Wilming et al. [32] 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 at the same time uncouple the influence of nitrogen source and pH value from each other. 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 [21], 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.

Conclusion and outlook

The *B. licheniformis* strain used in this study 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* very probably 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, 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 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 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. Other molecules such as nitrate can also act as electron and proton 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.

This study 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.

In future studies, an optimization of the product formation under the investigated fermentation conditions should 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.

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Conflict of interest The authors declare that they have no conflict of interest.

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