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Research Article

Analysis of intracellular metabolites of *Corynebacterium glutamicum* at high cell density with automated sampling and filtration and assessment of engineered enzymes for effective L-lysine production

Engineering of enzymes and pathways is generally required for the development of efficient strains for bioproduction processes. To this end, quantitative and reliable data of intracellular metabolites are highly desired, but often not available, especially for conditions more close to industrial applications, i.e. at high cell density and product concentration. Here, we investigated the intracellular metabolite profiles of an engineered L-lysine-producing *Corynebacterium glutamicum* strain and the corresponding wild-type strain to assess the impacts of deregulation of product inhibition of the key enzymes aspartate kinase and phosphoenolpyruvate carboxylase and to identify potentials for their further improvement. A bioreactor system with automated fast-sampling, filtration and on-filter quenching of the metabolism was used for a more reliable determination of intracellular metabolites in batch cultures with optical cell density (OD_{660}) up to 40. The L-lysine-producing strain showed substantially different metabolite profiles in the amino acid metabolism, including increased intracellular pool sizes in the L-lysine-, L-homoserine- and L-threonine pathways and decreased intracellular pool sizes for all other determined amino acids. By comparing data of in vitro inhibition of the engineered enzymes and determined intracellular concentrations of the inhibitors it was found that the inferred in vivo activities of these enzymes are still significantly below their in vitro maximums. This work demonstrates the usefulness of metabolic analysis for assessing the impact of engineered enzymes and identifying targets for further strain development.

Keywords: Intracellular metabolites / Fast filtration / *Corynebacterium glutamicum* / Lysine biosynthesis

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1 Introduction

The systematic and targeted optimization of enzymes and pathways in microorganisms for the improved production of industrially relevant biotechnological products is of high importance.

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Abbreviations: AK, aspartate kinase; CDW, cell dry weight; OD, optical density; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase

An increasing number of studies used metabolic engineering or synthetic biology approaches to design organisms with systematic modifications instead of using random mutagenesis for strain development [1–9]. The design and optimization of the gram-positive microorganism *C. glutamicum* for the production of L-lysine, or other industrially relevant amino acids is one of the prominent cases in this regard [4]. After sequencing the genome of *C. glutamicum* [10–13] targeted optimization approaches became more efficient.

One of the most important steps for the generation of an efficient lysine-producing mutant is the deregulation of the feedback inhibition of aspartate kinase (AK) in *C. glutamicum* [14].

This enzyme is concertedly feedback inhibited by the metabolites L-lysine and L-threonine and thus, the release of its allosteric inhibition is essential for the cells to overproduce lysine. Many lysine-producing mutants of *C. glutamicum* with deregulated inhibition of AK were generated by random mutagenesis [15, 16]. Recently, our group [17, 18] presented a rational approach for targeted design of minimally mutated variants of *C. glutamicum* containing only one point mutation for the release of feedback inhibition of AK. This strain was further optimized by rational deregulation of the allosteric inhibition of the phosphoenolpyruvate-carboxylase (PEPC) [19]. The phosphoenolpyruvate (PEP)—pyruvate—oxaloacetate node presents a very important node between glycolysis and the tricarboxylic acid cycle and is thus important for precursor supply during lysine biosynthesis [20]. PEPC in *C. glutamicum*, which catalyzes the reaction from PEP to oxaloacetate, is strongly feedback inhibited by the metabolites aspartate and malate [21]. Thus, the deregulation of this inhibition increased the flux by around 30% through PEPC and hereby improved lysine production [19].

Further targeted approaches include the optimization of NADPH supply for the lysine biosynthesis [22] and the design of regulatory proteins or riboswitches for dynamic regulation of metabolic pathways using intracellular metabolite as signal [23–25]. For all these approaches, it is of great interest to have reliable data on intracellular metabolite concentrations during cultivation, as these metabolites act as inhibitor or regulator within the cells.

However, the quantification of intracellular metabolites remains a highly complex task in order to avoid metabolite leakage [26–32] and in terms of rapidly quenching the cellular metabolism, especially at high cell density [33–35]. Recently, we have presented a new method for automated fast filtration with on-filter quenching to perform metabolite sampling with high accuracy within a short time scale [36]. It was shown that this automated method of sampling and sample treatment can greatly increase the quality of determination of intracellular metabolites in microorganisms like *Escherichia coli* by allowing a distinct separation of extra- and intracellular metabolites and making use of the advantages of process automation with regard to sampling time.

In this study, we comparatively investigated the lysine-producing strain *C. glutamicum* LP917, which contains engineered AK and PEPC with deregulated feedback inhibitions [17, 19] and the corresponding wild-type strain, to reveal the variations in the intracellular metabolite pools as consequence of the genetic modifications and to identify potentials for further enzyme and strain engineering. In particular, we determined the extend of feedback inhibition present in the modified enzymes in vivo, by combining data from Chen et al. [17] and Chen et al. [19] on the in vitro inhibition of the enzymes with the real intracellular concentrations of the inhibitory metabolites determined in this study. For the sampling and sample treatment for intracellular metabolite determination in this work, the optimized automated fast filtration method was used as described previously [36] and its suitability for the reliable determination of intracellular metabolite concentrations in *C. glutamicum* was investigated. This is the first study using automated sampling and fast filtration for the determination of intracellular metabolites in L-lysine-producing *C. glutam-*

icum at relatively high cell densities and controlled bioreactor cultivations.

2 Materials and methods

2.1 Chemicals and strains

Chemicals were purchased from Sigma Aldrich (Germany), Roth (Germany) or Merck (Germany) and were of analytical grade. The bacterial strains cultivated in this study were the wild-type strain *C. glutamicum* ATCC 13032 and the L-lysine-producing mutant strain *C. glutamicum* LP917, which has the point mutation Q298G in the *lysC* gene and N917G in the PEPC gene as described previously [17, 19].

2.2 Cultivation and medium

The cultivations were performed in a controlled bioreactor system with working volume of 2.5 L (Bioengineering, Switzerland) as described previously [36]. The batch cultivations were carried out at a temperature of 30°C, a stirrer speed of 1000 rpm and an aeration rate of 60 L/h. The pH was controlled at 7.2 using 2 M phosphoric acid and 25% ammonia solution for titration. The initial glucose concentration was 30 g/L and the exponential growth phase stopped after depletion of glucose. We used the fermentation medium as described previously [19], with the adaptations of omitting CaCl_2 and 3,4-dihydroxybenzoic acid and adding 1.1 g/L citric acid monohydrate.

2.3 Sampling, metabolite extraction and sample processing

The sampling for intracellular metabolites was performed by automated fast filtration and on-filter quenching, using a sampling system and sampling protocols developed previously [36]. The system allowed automated sampling, filtration and quenching with liquid nitrogen within less than 6 s. Three technical replicate samples were taken at each time point from one biological experiment, respectively. All technical replicate samples were processed separately. Metabolite extraction using boiling ethanol and further sample processing was performed as described before [36].

2.4 Analytics and calculations

Biomass concentrations were determined as optical density (OD) photometrically at 660 nm and gravimetrically as cell dry weight with three analytical replicates. The intracellular volume of *C. glutamicum* ATCC 13032 cells was determined by optical size measurement resulting in an intracellular volume of 1.44 $\mu\text{L}/\text{mg}$ CDW, which is in good accordance to literature values of 1.4 $\mu\text{L}/\text{mg}$ CDW for *C. glutamicum* [37, 38].

Organic acids, alcohols and glucose were measured by HPLC as described previously [36]. The determination of intra- and extracellular amino acids was performed using HPLC (Dionex, Thermo Scientific, Germany) as described previously [36] with

a slightly modified gradient. Briefly, AccQ-Tag method (Waters, USA) was used for derivatization and the analysis was performed using fluorescence detection. Derivatization efficiency was verified using alpha-aminobutyrate as internal standard. L-asparagine, L-cystine, L-serine and L-tryptophane could not be detected. The determination of PEP and malate was done using an HPIC system (ICS-3000, Dionex, Thermo Scientific, Germany) with conductivity detection. The separation was done with an IonPac AS11 column (Dionex, Thermo Scientific, Germany) using a flow rate of 350 $\mu\text{L}/\text{min}$ and a binary gradient with Eluent A being pure water and Eluent B being 100 mM KOH. The gradient was as follows: At 0 min 0.2% B; 34 min 11.4% B; 45 min 18.8% B; 67 min 24.4% B; 70 min 30.0% B; 72 min 100% B; 82 min 100% B; 83 min 0.2% B; 88 min 0.2% B. Ion suppression was done with an ASRS 2mm suppressor (Dionex, Thermo Scientific, Germany) with a current of 12 mA from 0 to 70 min and from 82.5 to 88 min and a current of 35 mA in between. For all chromatographic measurements, two analytical replicates were performed. The calculated 25% and 75% quartiles describe that 25% and respectively, 75% of the measured intracellular concentrations are below this value. The calculation was done using standard excel function as follows: $x_{[(n-1)p+1]} + ((n-1)p+1 - [(n-1)p+1])(x_{[(n-1)p+1]+1} - x_{[(n-1)p+1]})$, where n is the number of data points, p is the respective quartile with $0 < p < 1$ and x_n is the n th data point indexed from lowest to highest value.

3 Results and discussion

3.1 Automated fast filtration and on-filter quenching for metabolic analysis of *C. glutamicum* at elevated cell densities

To investigate the reliability of the automated fast filtration method for sampling of *C. glutamicum* cells at high cell densities, the influence of cell density on the filtration process was studied. For this purpose, automated fast filtration was carried out at different cell densities during batch fermentations of *C. glutamicum* ATCC 13032 and LP917. Figure 1 shows the filtrate volumes of samples and wash solution at different cell densities for both strains. The last data point in both diagrams of Fig. 1 was for samples during glucose starvation. The volumes of both the sample and wash solution remained quite constant throughout the fermentation process for both strains. There was a slight decrease of the wash solution volumes during glucose starvation for both strains. As shown by da Luz et al. [36], the constant filtrate volumes indicate accurate sampling without any filter clogging. Thus, the results showed that the sampling by automated fast filtration and on-filter quenching with a total sampling and processing time of less than 6 s can be used for reliable metabolite sampling from bioreactor cultures of *C. glutamicum* at elevated cell densities of at least an optical density of about 40.

Automated fast filtration and on-filter quenching was previously shown to allow fast sampling of intracellular metabolites in microorganisms with a reliable separation of intra- and extracellular metabolites [36]. The described sampling method was shown to deliver superior results for the investigation of intracellular amino acids in an *E. coli* strain as compared to the

Figure 1. Filtrate volumes of samples and wash solution at different cell densities for metabolite sampling using automated fast filtration directly from bioreactor cultures. (A) *C. glutamicum* wild type. (B) *C. glutamicum* LP917. Mean values and standard deviations were calculated from three technical replicates.

automated sampling with cold methanol–water quenching and subsequent centrifugation, which caused up to 75% loss of the intracellular amino acids due to metabolite leakage [36].

Previous metabolite studies of *C. glutamicum* using manual fast filtration as the sampling and sample treatment method, were all performed at low cell densities [26, 32, 39]. This study is the first study of intracellular metabolites in *C. glutamicum* that uses sampling by fast filtration at relatively high cell densities during controlled bioreactor cultivations. This allows the study of metabolic behavior in *C. glutamicum* during realistic cultivation conditions while avoiding metabolite leakage originating from cold shock as described previously for sampling using cold methanol–water quenching [28, 32, 36]. Moreover, the reliable separation of intra- and extracellular metabolites is of high importance, especially when aiming at the determination of the intracellular concentrations of products with high extracellular abundance, such as L-lysine.

3.2 Cultivation profiles of wild-type and lysine-producing strain LP917 of *C. glutamicum*

The investigation of intracellular metabolite profiles was carried out during batch cultures of *C. glutamicum*. Figure 2 displays the cultivation profiles of these batch fermentations. The biomass growth of both strains was almost identical, with a slightly longer lag-phase of the wild-type strain (Fig. 2A). In both batch

Figure 2. Cultivation profiles of *C. glutamicum* wild type and LP917 during batch cultures. (A) Biomass growth as optical density OD₆₆₀. Means and standard deviations of optical density were calculated from three technical replicates. (B) Extracellular L-lysine concentration. Mean values were calculated from two analytical replicates and the whiskers show the upper and lower value, respectively.

cultivations, the growth was finally limited by the substrate glucose contained in the medium. The specific growth rates in the exponential phase were calculated as $\mu = 0.245 \text{ h}^{-1}$ for the wild type and $\mu = 0.251 \text{ h}^{-1}$ for *C. glutamicum* LP917, respectively. The wild-type *C. glutamicum* reached a higher maximum cell density of OD = 40.5 compared to OD = 30.6 of the strain LP917, at the end of the batch fermentation. This was due to the higher biomass specific glucose consumption rate of the strain LP917 resulting from the production of L-lysine. The specific glucose consumption rates of the wild-type strain and strain LP917 were $q_s = 0.49 \text{ g}_{\text{Gluc}} (\text{g}_{\text{CDW}} \text{ h})^{-1}$ and $q_s = 0.64 \text{ g}_{\text{Gluc}} (\text{g}_{\text{CDW}} \text{ h})^{-1}$ during the exponential growth phase, respectively.

Figure 2B shows the concentrations of extracellular L-lysine secreted during the cultivation. The L-lysine-producing strain LP917 reached a final concentration of 28.3 mM L-lysine after the consumption of 164 mM glucose which corresponds to an L-lysine yield of 172.5 mmol/mol of glucose.

To accommodate the slightly different lag-phases of the cultivations, the sampling for intracellular metabolites was performed at similar biomass concentrations instead of similar fermentation times to obtain samples from the same fermentation stage. The sample times during the cultivations are given in Table 1. For each cultivation, six sampling times were defined, starting at the early exponential phase with five data points during the growth phase and one data point approximately 30 minutes after the depletion of glucose. With these sampling points

Table 1. Time points for intracellular metabolite sampling using automated fast filtration

Sample	Wild type		LP917	
	Time [h]	OD ₆₆₀ [AU]	Time [h]	OD ₆₆₀ [AU]
1	13.4	4.9	12.3	4.5
2	16.1	9.1	14.3	8.5
3	18.2	15.2	17.0	16.3
4	19.3	20.1	17.7	20.7
5	21.2	31.9	20.0	30.1
6	22.8	38.7	20.6	30.6

it was possible to investigate the whole process including the growth and glucose limitation phases.

3.3 Intracellular metabolite profiles of wild type and *C. glutamicum* LP917

To investigate the influences of the genetic modifications introduced into the strain LP917 on its metabolism, intracellular metabolites of both, the wild-type strain and the lysine producer LP917 were determined and compared to each other. A simplified overview of the amino acid pathways of *C. glutamicum* is displayed in Fig. 3. Figures 4 and 5 show the intracellular concentration profiles for some important amino acids, PEP and malate. The profiles of all other determined intracellular amino acids can be found in the Supporting Information. In the following sections, the metabolites are grouped by their position in the metabolism for discussion.

3.3.1 Aspartate and lysine metabolism

Figure 4A displays the concentration profiles of intracellular L-lysine for both strains. The L-lysine concentration of the wild type was at $12.8 \mu\text{mol/g CDW}$ at the beginning of the exponential phase. Afterward, the lysine concentration dropped down rapidly and remained at about $4\text{--}5 \mu\text{mol/g CDW}$ throughout the rest of the cultivation. No influence of the glucose depletion at the last sample point was determined. The strain LP917 showed substantially higher intracellular L-lysine levels, than the wild type. Again, the L-lysine level was elevated at $87.0 \mu\text{mol/g CDW}$ in the beginning of the exponential phase. Subsequently, the concentration decreased to lower levels and remained in the range of $43.8\text{--}55.9 \mu\text{mol/g CDW}$ during the later course of fermentation. This observation of elevated intracellular L-lysine levels at the beginning of the L-lysine synthesis and lower, rather constant L-lysine levels throughout the rest of the production phase was also described by Krömer et al. [39] for another L-lysine-producing *C. glutamicum* strain. Krömer et al. [39] assumed a delayed L-lysine secretion with intracellular accumulation during the first period of the production, which could be confirmed in this study. Since the lysine production is feedback inhibited by the product L-lysine, this observation shows that one important challenge for further optimization of strain LP917 is to improve the L-lysine secretion into the extracellular medium.

The intracellular L-aspartate concentrations (Fig. 4B) showed similar concentration profiles in the wild type and the strain

Figure 3. Simplified overview of the amino acid metabolism in *C. glutamicum*.

LP917 throughout the exponential phase. Interestingly, the L-aspartate levels showed opposing behavior after the depletion of glucose, where the L-aspartate level of the wild type decreased while the L-aspartate level of the strain LP917 increased considerably from 28.6 to 83.4 $\mu\text{mol/g}$ CDW. This behavior was also observed for L-threonine and glycine (see Fig. 4C and D) and may be a result of a possible consumption of extracellular glycine present for LP917 during glucose limitation, which then caused rising intracellular levels of these compounds.

3.3.2 Glutamic acid metabolism

The intracellular concentrations of L-glutamic acid (Fig. 4E) and L-glutamine (Supporting Information Fig. S1 and S2) were on relatively high levels and with only slight changes throughout the cultivations. Both amino acids were at higher levels in the wild type as compared to strain LP917. The intracellular concentrations of L-glutamic acid in the wild type were around 440 $\mu\text{mol/g}$ CDW and around 320 $\mu\text{mol/g}$ CDW in strain LP917, respectively. This difference of 120 $\mu\text{mol/g}$ CDW is likely to originate from the increased L-lysine production in strain LP917, as proposed by Krömer et al. [39].

The intracellular L-proline concentration (Fig. 4F) at the beginning of the exponential phase was nearly identical in both strains with 259 $\mu\text{mol/g}$ CDW for the wild type and 253 $\mu\text{mol/g}$ CDW for strain LP917, respectively. During the cultivations,

the L-proline levels of both strains decreased to well below 100 $\mu\text{mol/g}$ CDW at the end of the cultivations, while the intracellular L-proline of strain LP917 decreased significantly faster than that of the wild type. The relatively high intracellular L-proline levels in both strains, as well as their decreasing profiles indicate strong osmotic stress of the microorganisms especially at the beginning of the exponential phase, which decreased during the rest of the cultivations. This is due to the role of L-proline as a compatible solute. It is known in the literature that in osmotic stress situations, *C. glutamicum* accumulates intracellular L-proline, as well as L-alanine and L-valine to compensate the osmotic stress [40]. In conjunction with the concentration profiles of L-alanine and L-valine (see below), this suggests the need of further medium and process optimization to reduce the osmotic stress level during cultivation. The faster decrease of intracellular L-proline in strain LP917 suggests that the osmotic stress level was reduced due to the modified product spectrum of the L-lysine-producing strain.

3.3.3 Pyruvate metabolism

Similar to the concentration profile of L-proline the intracellular concentration profiles of L-alanine and L-valine also showed decreasing trends as shown in Fig. 4G and H. Both amino acids showed relatively high concentrations at the beginning of the exponential phase, with L-alanine at $41 \mu\text{mol/g}$ CDW for the

Figure 4. Concentration profiles of intracellular metabolites during batch cultures of *C. glutamicum* wild type and *C. glutamicum* LP917. The sampling points are displayed by the biomass concentrations according to Table 1. Mean values and standard deviations were calculated from three technical replicate samples.

wild type and 28 $\mu\text{mol/g CDW}$ for strain LP917 as well as L-valine at 27 $\mu\text{mol/g CDW}$ and 7.7 $\mu\text{mol/g CDW}$, respectively. Both amino acids decreased during cultivation below 10 $\mu\text{mol/g CDW}$. As mentioned above, the role of L-alanine and L-valine as compatible solutes similar to L-proline is likely to be responsible for the determined intracellular concentration profiles [40].

3.3.4 PEP and malate

The intracellular concentration profiles of PEP are shown in Fig. 5A. At the beginning of the exponential phase, the PEP

concentration of strain LP917 was similar to that of the wild type at around 0.1 $\mu\text{mol/g CDW}$. However, during the middle and late exponential phase it decreased considerably to only 0.02 $\mu\text{mol/g CDW}$. This lower intracellular PEP concentration in strain LP917 compared to the wild type may be a result of the modified PEPC in LP917. Chen et al. [19] showed that the flux from PEP to oxaloacetate through the PEPC was increased by about 30%, due to the genetic modification. Thus, a reduced intracellular PEP concentration was to be expected. The intracellular malate concentrations were slightly lower in strain LP917, than in the wild type, as shown in Fig. 5B.

Figure 5. Concentration profiles of intracellular metabolites during batch cultures of *C. glutamicum* wild type and *C. glutamicum* LP917. The sampling points are displayed by the biomass concentrations according to Table 1. Mean values and standard deviations were calculated from three technical replicate samples.

3.4 Comparison of intracellular amino acid levels of *C. glutamicum* wild type and LP917

In most of the cases, the trends of intracellular amino acid profiles of both strains were rather similar. However, the absolute concentrations of amino acids varied in different amounts. To quantify the differences between both strains, for each compound the relative difference of the intracellular amino acid concentration δ was determined as the sum of the differences between the strains for all sampling points during the growth phase and normalized by the mean concentration of the compound in the wild type. Thus, a positive value of δ indicates an overall higher concentrated amino acid profile in the strain LP917 compared to the concentration profile of the wild type.

Figure 6 shows the relative differences δ for all determined amino acids. As can be seen here, most amino acids were lower concentrated in the strain LP917 than in the wild type. However, exclusively the amino acids of the lysine-, homoserine- and threonine metabolism were higher concentrated in strain LP917. DL-diaminopimelate, L-homoserine and L-methionine were about 2.5–4 times higher, while L-threonine and L-lysine showed an even stronger increase with $\delta_{thr} = 7.3$ and $\delta_{lys} = 40.2$, respectively. The amino acids glycine and L-isoleucine showed rather slight increase with $\delta_{gly} = 1.7$ and $\delta_{ile} = 0.5$, respectively. These less pronounced increases of glycine and isoleucine can be associated with the secretion of these compounds into the fermentation medium during the cultivation of strain LP917 (data not shown). The intracellular concentrations of L-aspartate were unchanged among the two strains, represented by a $\delta_{asp} = 0.1$.

The increased intracellular concentrations of all amino acids of the lysine-, homoserine- and threonine metabolism along with the unchanged aspartate concentration suggest a direct influence of the deregulation of the feedback inhibition of the AK by Chen et al. [17]. The genetic modification resulted in an upregulation of the L-lysine biosynthesis pathway, as well as the connected pathways for L-homoserine and L-threonine. Concurrently to this increase of intracellular concentrations within the downstream pathways of L-aspartate, other intracellular amino acid concentrations, i.e. of the L-glutamic acid- or pyruvate metabolism decreased. This is potentially to compensate for the higher resource demand in the L-lysine biosynthesis. Chen et al. [19] showed previously that the metabolic flux to pyruvate was decreased in the mutant strain LP917.

Interestingly, the intracellular concentration of L-aspartate was not changed, while Chen et al. [19] showed that the flux from PEP to oxaloacetate was increased and thus, a higher supply of L-aspartate was generated. The observation, that the intracellular pool size of L-aspartate was unchanged, while fluxes increased, may be a result of the strong regulation of L-aspartate within the metabolism and is a hint for the high importance of this metabolite and its intracellular pool size in metabolic regulation.

The results show, that not only the metabolite fluxes in these pathways increased, which resulted in the production of the target product, but also the intracellular concentrations. This is particularly important, when aiming at dynamic control and regulation of biosynthetic pathways using intracellular metabolites as signal [23, 25].

We are aware of the fact, that pool sizes of free intracellular amino acids are low compared to the metabolite fluxes. Nonetheless, the results show that not only the metabolite pools of the target product L-lysine and its precursor DL-diaminopimelate were increased by the genetic modifications, but also the amino acids of the related pathways of L-homoserine- and L-threonine. For further improvement of the L-lysine production of the strain LP917, the increased intracellular concentrations of these metabolites should be considered, as they take part in the feedback regulation of L-lysine related pathways.

3.5 Inhibition of aspartate kinase by intracellular lysine and threonine

As mentioned before, the AK is a key enzyme for the regulation of the lysine biosynthesis (see Fig. 3). The wild-type AK is feedback inhibited by the metabolites L-lysine and L-threonine [41]. To deregulate the feedback inhibition, the strain LP917 has a modified AK with a strongly reduced sensitivity to these inhibitors, which enables this strain to overproduce L-lysine [17]. However in Chen et al. [17], the enzyme inhibition profile was only investigated in vitro. It is important to investigate the influence of the real intracellular concentrations of the inhibitors as determined in this study, on the inhibition of the AK's of the wild type and the strain LP917, to gain a better understanding about the metabolic behavior of the L-lysine production strain.

In Fig. 7A, the in vitro inhibition profiles of the wild-type AK are shown. As demonstrated by Chen et al. [17], the wild-type AK showed only slight inhibition by L-lysine and almost no inhibition by L-threonine. However, the combination of both

Figure 6. Comparison of intracellular amino acid levels of *C. glutamicum* wild type and LP917. The relative difference δ describes the normalized overall difference for all sampling points during the exponential growth phase: positive values represent higher intracellular levels in the lysine-producing strain LP917 compared to the wild type. Error bars represent the normalized cumulated errors of the five sampling points during exponential growth.

Figure 7. In vitro inhibition profiles of aspartate kinase for lysine, threonine and concerted inhibition of both amino acids according to [17]. The shaded areas represent the in vivo concentration ranges of intracellular lysine (hatched) and threonine (diagonal lines) determined in this study (25% to 75% quartile of the concentrations determined during exponential growth). (A) *C. glutamicum* wild type. (B) *C. glutamicum* LP917.

inhibitors resulted in a strong inhibition of the enzyme. The shaded areas in Fig. 7A represent the intracellular concentration ranges during exponential growth, determined in this study (25 to 75 % quartile of the measured concentrations). It can be seen clearly, that the wild-type AK was strongly feedback inhibited in vivo by the intracellular inhibitors with a residual relative activity of only 10 %.

Figure 7B shows the in vitro inhibition profiles of the modified AK which is present in the *C. glutamicum* strain LP917, determined by Chen et al. [17]. The feedback inhibition was strongly reduced, especially in the case of concerted inhibition by L-lysine and L-threonine. However, the analyzed range of the inhibition

profile in vitro was lower compared to the observed real intracellular concentrations of the inhibitors (shaded areas represent the 25% to 75% quartile of the measured concentrations during exponential growth). We assume here by extrapolating the in vitro inhibition profile that a residual relative activity of less than 60% is realistic. This comparison of the true intracellular inhibitor concentrations with the inhibition profiles determined in vitro by Chen et al. [17] shows that to achieve a complete deregulation of the feedback inhibition of the AK and thus using its full potential, further strain and enzyme modifications are necessary and can result in a higher overproduction of L-lysine. Beside an even stronger deregulation of the feedback inhibition of the AK of strain LP917, the improvement of the L-lysine secretion turned out to be the most important and most promising optimization step for further improvement of the L-lysine production of *C. glutamicum* LP917. An improved L-lysine secretion should result in lower intracellular concentrations and thus in less pronounced inhibition of the AK.

3.6 Inhibition of the PEPC by intracellular L-aspartate and malate

Similar to the investigation of the AK inhibition in the previous section, the PEPC of the wild-type strain and the L-lysine producer LP917 were investigated. As mentioned before, the PEPC catalyzes the reaction from PEP to oxaloacetate and thus plays a key role in the supply of oxaloacetate as precursor for the L-lysine biosynthesis [19, 20]. However, the wild-type PEPC is feedback inhibited by the metabolites L-aspartate and malate. Besides the modified AK, the strain LP917 has a modified PEPC with deregulated feedback inhibition, by which Chen et al. [19] were able to increase the L-lysine production of strain LP917 significantly.

Figure 8A shows the in vitro inhibition profiles of the wild-type PEPC, as determined by Chen et al. [19]. The wild-type PEPC was strongly inhibited by both metabolites, the L-aspartate and the malate. For similar concentrations of both metabolites, the inhibition by L-aspartate was slightly stronger. Indeed, the intracellular concentrations determined in vivo within this study (shaded areas represent the concentration ranges of the 25% to 75% quartiles of the determined intracellular concentrations during exponential growth) showed, that L-aspartate was the main inhibitor, since the intracellular malate concentrations were relatively low around 2 mM.

Figure 8. In vitro inhibition profiles of phosphoenolpyruvate carboxylase for Aspartate and malate, according to [19]. The shaded areas represent the in vivo concentration ranges of intracellular Aspartate and malate determined in this study (25% to 75% quartile of the concentrations determined during exponential growth). (A) *C. glutamicum* wild type. (B) *C. glutamicum* LP917.

Figure 8B displays the in vitro inhibition profiles of the modified PEPC as determined by Chen et al. [19]. The feedback inhibition of the PEPC was significantly reduced for both inhibitors compared to the wild-type PEPC. As opposed to the wild-type PEPC, for similar intracellular concentrations of the two inhibitors, the modified PEPC inhibition by malate was stronger than the inhibition by L-aspartate. However, as seen in the shaded areas (25% to 75% quartiles), the intracellular concentration range of L-aspartate is much higher compared to malate. Thus the main inhibitor is L-aspartate instead of malate. This underlines the necessity to compare the inhibition profiles with the real intracellular concentrations determined in vivo to clarify, which compound has the stronger inhibiting effect on the enzyme. For the further optimization of the PEPC in *C. glutamicum*, the presented data show the importance of further releasing the feedback inhibition by L-aspartate, since this metabolite reduced the relative enzyme activity by about 20% in the strain LP917.

4 Concluding remarks

Automated fast filtration and on-filter quenching was shown for the first time to allow a more accurate determination of intracellular metabolites in *C. glutamicum* at relatively high cell densities during controlled bioreactor cultivations. In this study, the sam-

pling method was applied to *C. glutamicum* cultures with optical cell densities up to 40 (OD_{660}), while reliably separating intra- and extracellular metabolites and avoiding metabolite leakage during quenching.

The detailed investigation of intracellular metabolite profiles during batch cultivation of an L-lysine-producing *C. glutamicum* strain and the comparison to the metabolite profiles of the original wild-type strain enabled useful insights into the metabolism of these strains. The influences of the genetic modifications were clearly visible in the metabolite pool sizes and their profiles throughout the cultivations. We described possible process improvement, i.e. regarding the reduction of osmotic stress level by media optimization. The fact, that the upregulation of certain pathways (i.e. the L-lysine-, L-homoserine- and L-threonine pathways) results not only in higher fluxes, but also in higher pool sizes of certain metabolites, and the quantitative knowledge about these pool sizes are of high importance when aiming at the dynamic regulation and control of metabolic pathways using intracellular metabolites as signal molecules. Furthermore the results showed that for the confirmation of successful deregulation of allosteric inhibitions within the metabolism, it is highly important to quantify the intracellular metabolites that act as inhibitors, to determine the in vivo activities of the deregulated enzymes. The presented results in this work show the necessity for further genetic optimization of the L-lysine-producing strain. Especially, the improvement of the L-lysine secretion is of high importance, in order to reduce its intracellular concentration.

Practical application

In this work, we present results of intracellular metabolite analysis using automated fast sampling and filtration for bioreactor culture of an L-lysine-producing *Corynebacterium glutamicum* strain at relatively high cell density and their application for assessment of efficiency of engineered enzymes. The suitability of the method for sampling and filtration of culture broth up to an optical density of about 40 directly from bioreactor was demonstrated. The investigation of intracellular amino acid pools showed strong influence of engineered enzymes on the pool sizes of the L-lysine-producing strain. Furthermore, the estimation of in vivo enzyme activities using in vitro inhibition profiles in connection with the measured intracellular concentrations is of high importance in order to assess the degree of deregulation of engineered enzymes. The method and findings have practical applications in the rational engineering of enzymes and metabolic pathways, where intracellular metabolites are used as signal molecules and in the technology development for analysis of intracellular metabolites in general.

The authors have declared no conflict of interest

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