1 Introduction

1,5-diaminopentane, also known as cadaverine, is an important industrial platform chemical with a variety of applications including the production of polyamides or polyurethanes, chelating agents, or additives. In particular, it is relevant for bio-polyamides derived from renewable feedstocks to replace conventional polyamides from petrochemical routes. Polyamides are widely used due to their extreme durability and strength, e.g. in the automotive industry or in high-value consumer products. With annual production volume of 3,500,000 metric tons, they display an important fraction in the area of polymers with increased material properties. Since their production from petrochemically derived monomers suffers from the shortage of fossil fuels, rising oil price, escalating CO_2 production as well as low eco-efficiency, biotechnological production from renewable resources arises as promising alternative with an enormous ecological and economical potential (Steinbüchel 2005, Willke and Vorlop 2004). Polymerization of diaminopentane derived from microbial biosynthesis with appropriate bio-blocks such as succinate from microbial fermentation or sebacic acid from natural plant oils provides the completely bio-based polyamides PA 5.4 and PA 5.10, respectively. In contrast to polylactic acid or polyhydroxyalkanoates, which are the first industrial examples of biotechnologically produced polymers (Flieger et al. 2003), diaminopentane-based polyamides exhibit excellent, well-known material properties (Carothers 1938). From a metabolic viewpoint, diaminopentane is formed directly from lysine by decarboxylation. Thus *C. glutamicum*, a Gram-positive soil bacterium, appears to be a promising production organism since it is used for the industrial production of more than 1,000,000 metric tons of L-lysine per year. Pioneering studies successfully demonstrated diaminopentane production in *C. glutamicum* by heterologous expression of the *cadA* gene, encoding lysine decarboxylase from *E. coli* (Mimitsuka et al. 2007, Tateno et al. 2009). The introduction of a lysine decarboxylase gene in *C. glutamicum* displays the key modification for the production of diaminopentane, which is not a natural product of this organism.

An important criterion for the establishment of bio-based processes as alternative to petrochemistry is the economic competitiveness with conventional techniques, which demands for efficient microbial strains overproducing diaminopentane. The experience of the past clearly shows that a superior production strain with a high yield, a high productivity, and a high titer requires substantial modifications at different key points of the metabolism, which have to be identified by careful investigation of the underlying metabolism (Wittmann 2010). In the beginning, efficient production strains were created by applying classical random mutagenesis using UV light or chemical mutagens and subsequent strain selection (Nakayama et al. 1978). By this approach, remarkable production properties were achieved e.g. for lysine (Leuchtenberger et al. 2005), which are, however, typically linked to extensive

fermentation times, weak stress tolerance, and auxotrophies (Ohnishi et al. 2002). Since the advent of recombinant DNA technology, systems-oriented studies, based on the available genome sequence and post genomic techniques, serve as powerful strategy for rational strain optimization (Lee et al. 2005, Wendisch 2006, Wittmann and Becker 2007). The complexity of the underlying network hereby requires an interaction of different biochemical, genetic, physiological, and mathematic techniques, whereby transcriptome, proteome, metabolome, and fluxome analysis provide useful insights into the metabolism. Based on a detailed characterization, it is then possible to construct high-efficient production strains by introduction of directed genetic modifications. Major targets leading to increased production properties typically comprise the release from undesired regulatory phenomena, the elimination of by-products, the optimization of precursor or cofactor supply, and the optimization of product export.

2 Objectives

The aim of the present work was the generation of a diaminopentane-producing *C. glutamicum* strain by heterologous expression of a lysine decarboxylase encoding gene and subsequent optimization of the production properties by rational strain optimization using a systems-wide metabolic engineering approach. Thereby, systems biology techniques should be used to unravel the physiological state of the modified strains and to derive strategies for further optimization of the diaminopentane production. Since the production of diaminopentane is closely connected to the central metabolism and involves various supporting pathways for the supply of precursors and reducing power, central metabolic reactions arise as promising targets. The different strategies focused on engineering of precursor supply, diaminopentane biosynthesis, by-product formation, TCA cycle, and NADPH supply. The identification of the diaminopentane export system in *C. glutamicum*, which has not been described to date, provides another starting point for optimization displays. Regarding the charged nature of diaminopentane, a non-natural metabolite in *C. glutamicum*, the existence of an active transport system appears very likely. In detail, investigation of the created mutants should be performed by comparative cultivation experiments, combined with data from different omics techniques including transcriptomics, metabolomics, and fluxomics. The final objective was the creation of a wild type-based diaminopentane hyper-producer by combining the beneficial modification identified in this work. In order to ensure an efficient diaminopentane production, this tailor-made cell factory should reach a high carbon yield, a high space-time yield, and a high final diaminopentane titer. Additionally, the lack of any by-product formation would facilitate the downstream processing and thus reduce the production costs. Furthermore, this work should show the enormous potential of synthetic metabolic engineering carried out at the systems-wide level and encourage the generation of superior strains for the bio-based production of further chemicals and fuels.

3 Theoretical Background

3.1 Bio-based Production of 1,5-Diaminopentane - a Polyamide Monomer

1,5-diaminopentane, also known as cadaverine, is an important platform chemical with a variety of industrial applications including the production of polyamides or polyurethanes, chelating agents, or additives. From a chemical viewpoint, diaminopentane is a linear aliphatic diamine with five carbon atoms. More than 100 years ago, it was isolated and described for the first time during systematic experiments on the bacterial putrefaction of human cadavers, providing rather directly its trivial name cadaverine (Brieger 1885). Diaminopentane is a minor member of the group of biogenic polyamines that is formed *in vivo* through decarboxylation from lysine by lysine decarboxylase (EC 4.1.1.18). In the past, it was observed in a few microorganisms (Tabor and Tabor 1985). However, lysine decarboxylase as major forming enzyme is encoded in the genome of various bacteria suggesting a probably broader distribution (Takatsuka et al. 2000). Interestingly, diaminopentane can modulate cellular processes possibly due to its positive charge at physiological pH (Igarashi and Kashiwagi 2010). As an example, it protects microbial cells from superoxide stress (Chattopadhyay et al. 2003, Kang et al. 2007, Kim et al. 2006b, Tkachenko 2004) as well as from antibiotics (Samartzidou and Delcour 1999, Tkachenko et al. 2006, Tkachenko et al. 2009). The vital interest in bio-based supply of diaminopentane has strongly stimulated the research on developing engineered microbial production strains. The most promising development for bio-based supply of diaminopentane is microbial production using genetically engineered microorganism like *Corynebacterium glutamicum* (Mimitsuka et al. 2007, Tateno et al. 2009) or *Escherichia coli* (Qian et al. 2010). In addition, enzymatic production of 1,5-diaminopentane from lysine-rich media with external addition of lysine decarboxylase has been proposed (Mimizuka and Kazami 2002).

3.1.1 Replacement of chemical polymer synthesis

Regarding the application possibilities, diaminopentane is particularly relevant as key building block for the production of novel bio-polyamides. These bio-polyamides, derived from renewable feedstocks, are supposed to replace conventional polyamides currently derived from petrochemical routes which suffer from shortage and rising prices of the fossil resources as well as low eco-efficiency (Figure 3-1). In addition, escalating CO_2 production and global warming are major drivers of a new bio-economy and lead to an increased interest in biotechnological processes for the production of chemicals (Steinbüchel 2005,

Willke and Vorlop 2004). In the field of polymers, comprising a significant fraction of today's chemical products, polylactic acid (PLA) and polyhydroxyalkanoate (PHA) are the first industrial examples of biotechnologically derived materials as an alternative to petroleum-based products (Flieger et al. 2003). PHA, beneath e.g. polyhydroxybutyrate (PHB), belongs to the class of linear polyesters and is produced in nature by bacterial fermentation of sugars or lipids. Microorganisms like *Ralstonia eutropha* produce these polyesters to store carbon and energy in response to physiological stress conditions (Katoh et al. 1999, Jacquel et al. 2008, Yu et al. 2002). Lactate, the building block of PLA, is a classical product of *Lactobacillus* strains which have been widely used for the production of enantioselective D- or L-lactate (Benthin and Villadsen 1995, Kylä-Nikkilä et al. 2000). The material properties of these polymers, however, resemble those of low-cost standard polymers such as polyethylene. In the area of polymers with advanced material properties, production is nowadays still based on petrochemically derived monomers. This holds also true for polyamides used e.g. in the automotive industry or in high-value consumer products like textiles, carpets and sportswear, due to their extreme durability and strength. The most prominent products, polyamides PA 6 and PA 6.6, have an annual market volume of about six million tons. Biotechnological production of these polyamides arises as a promising alternative, as it possesses enormous ecological and economical potential. In this regard, the fermentative production of diaminopentane as a monomer building block has come into focus. Polyamides, examples being nylons, aramids or polyaspartate, are polymers of monomeric diamines and dicarboxylic acids, joined by peptide bonds. Using diaminopentane derived from microbial biosynthesis, polymerization with appropriate bio-blocks such as succinate from microbial fermentation (Hong et al. 2004, Oh et al. 2009), or sebacic acid from natural plant oils (Ogunniyi 2006) allows the production of completely bio-based polyamides (Figure 3-1). Moreover, polyamides based on diaminopentane reveal excellent, long-known material properties (Carothers 1938). This opens novel markets, e.g. in the automotive industry or in high value consumer end products, beyond current applications of the existing low performance biopolymers.

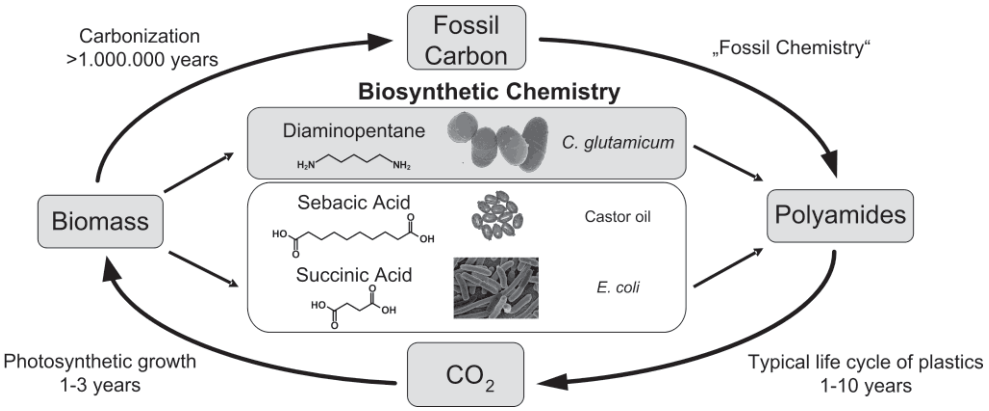

Figure 3-1: Circular carbon flow concerning polyamide synthesis. Shown is the usage of biomass in a fossil form (outer circle) and the biosynthetic chemistry as alternative route.

3.1.2 Metabolism of diaminopentane in *Escherichia coli* and *Corynebacterium glutamicum*

From a metabolic viewpoint, diaminopentane is formed directly from lysine by decarboxylation. As the metabolism of diaminopentane has been studied extensively in bacteria, there exists a rather profound knowledge on the biosynthesis, degradation, import, and export of this compound. The industrial workhorse *E. coli*, which comprises the entire pathway set for the biosynthesis of diaminopentane, appears as a promising host for industrial production. The *E. coli* diaminopentane biosynthetic pathway branches off the tricarboxylic acid (TCA) cycle, starting from oxaloacetate. It then proceeds via ten successive steps, requiring as additional building blocks one pyruvate molecule, two ammonium molecules, and four nicotinamide adenine dinucleotide phosphate (NADPH) molecules (Michal 1999) (Figure 3-2). Hereby, the second step, the phosphorylation of aspartate to aspartyl phosphate, is catalyzed by three isoenzymes. Aspartokinase I (ThrA) (Katinka et al. 1980) and aspartokinase II (MetL) (Zakin et al. 1983) are two bifunctional enzymes that also catalyze the conversion of the branch point intermediate aspartate-semialdehyde to homoserine and thereby channel carbon into the split pathway to threonine, methionine and isoleucine. Aspartokinase III (LysC) (Cassan et al. 1986) is specific to the diaminopentane biosynthetic pathway. Diaminopimelate, an essential constituent of cell wall peptidoglycan, is another branch point intermediate, which is directed to the diaminopentane branch by dihydropicolinate synthase. Diaminopentane biosynthesis in *E. coli* is regulated by its precursor lysine at various steps, encoded by the *lysC*, *asd*, *dapA*, *dapB*, *dapD* and *lysA* genes, through transcriptional repression as well as through feedback inhibition at the steps catalyzed by the enzymes LysC and DapA (Qian et al. 2010) (Figure 3-2). For the final conversion from lysine to diaminopentane, *E. coli* possesses two variants of lysine decarboxylase, CadA (Meng and Bennett 1992, Watson et al. 1992) and LdcC (Yamamoto et

al. 1997), which both require pyridoxal phosphate as cofactor. The expression of the degradative enzyme CadA is induced at low pH in the presence of lysine and achieves maximal levels under anaerobic conditions (Neely and Olson 1996). By excretion of diaminopentane, the pH of the extracellular medium is neutralized (Meng and Bennett 1992, Watson et al. 1992). The CadA expression is positively regulated by the gene product of the upstream gene *cadC*, mediating acid induction (Auger and Bennett 1989, Watson et al. 1992), and negatively regulated by the product of the distant *cadR* gene (Popkin and Maas 1980, Tabor et al. 1980). As there is almost no diaminopentane detectable in *E. coli* growing in minimal medium at neutral pH, the *cad* operon is effectively silenced under these conditions (Hafner et al. 1979). The second, probably constitutively expressed lysine decarboxylase encoded by *ldcC* is active over a broad pH range with an optimum at pH 7.6 (Lemonnier and Lane 1998, Yamamoto et al. 1997). However, *ldcC* expression from its natural promoter is very weak for cells growing in different media (Lemonnier and Lane 1998). Beside biosynthesis, the level of diaminopentane in *E. coli* is also regulated by uptake, export, and degradation (Soksawatmaekhin et al. 2004). The diaminopentane transport protein CadB has both, a diaminopentane uptake activity, dependent on the proton motive force, and, while acting as diaminopentane-lysine antiporter, a diaminopentane excretion activity. For diaminopentane utilization and degradation, there are different pathways in *E. coli* (Figure 3-2). In addition to *speE*, encoding putrescine/cadaverine aminopropyltransferase (Bowman et al. 1973) and *speG*, encoding spermidine acetyltransferase, a putative diamine acetyltransferase, YgjG (Haywood and Large 1985), has shown to be able to transaminate diaminopentane (Samsonova et al. 2003). Besides, PuuA, the first enzyme of the putrescine utilization pathway, catalyzes the γ-glutamylation of diaminopentane (Kurihara et al. 2008).

In terms of biotechnological diaminopentane production, a recent study describes the conversion of a wild type *E. coli* strain into a remarkable cell factory for diaminopentane using metabolic engineering (Qian et al. 2010). For that purpose, modifications at different levels of the metabolism were implemented in a *lacI*-deletion strain, enabling a strong, constitutive expression of target genes under the *tac* promoter (Park et al. 2007a). The modifications comprise the overexpression of *cadA* under control of the *tac* promoter on a multi-copy plasmid to increase the conversion of lysine to diaminopentane, as well as the chromosome-based overexpression of *dapA* via the strong *trc* promoter to redirect carbon from the competing threonine pathway towards the lysine pathway. Since diaminopentane utilization and degradation pathways likely reduce diaminopentane yield, these pathways were disrupted by deletion of the genes *speE*, *speG*, *ygjG* and *puuA*, coding for enzymes which exhibit possible diaminopentane degradation activity. Additionally, the putrescine importer PuuP, which might also be able to import diaminopentane due to structural

similarities, was inactivated. The *E. coli* strain, carrying the specified modifications, produced 212 mmol diaminopentane per mol glucose.

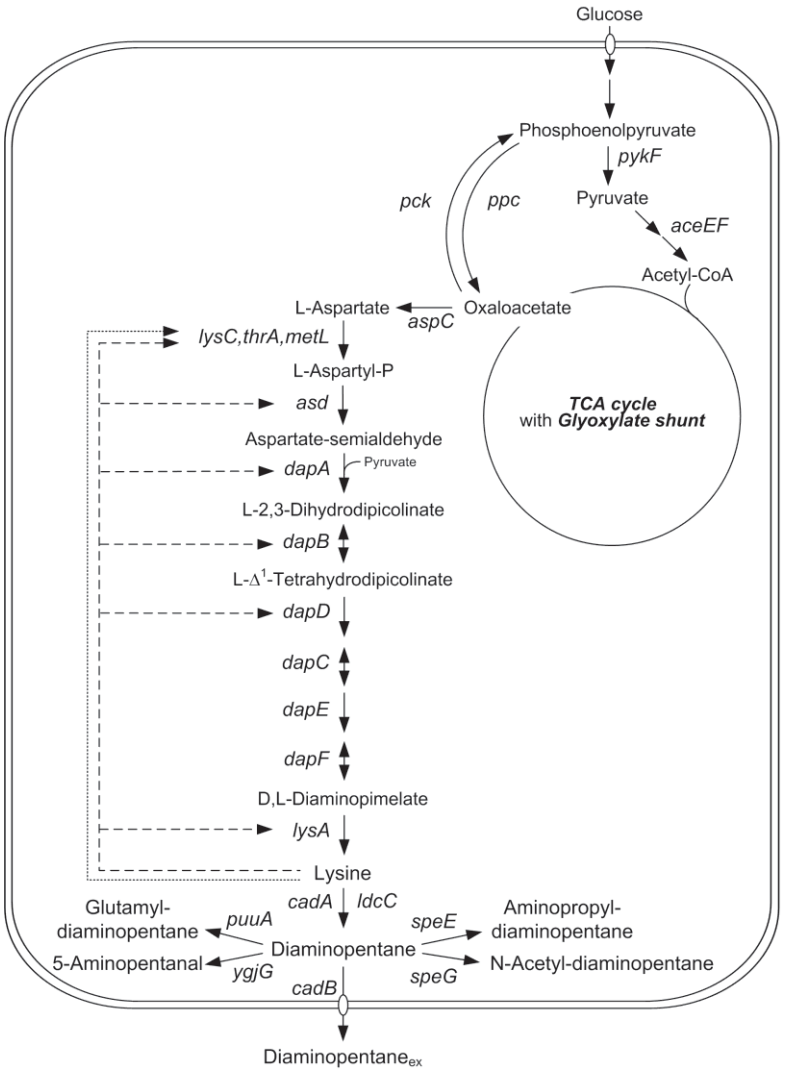

Figure 3-2: Overview of the pathways involved in diaminopentane metabolism in *E. coli*. Dotted lines indicate feedback inhibition, and dashed lines indicate transcriptional repression. The enzymes encoded by the genes are *ppc* phosphoenolpyruvate carboxylase, *pck* phosphoenolpyruvate carboxykinase, *pykF* pyruvate kinase, *aceEF* pyruvate dehydrogenase, *aspC* aspartate aminotransferase, *lysC* aspartokinase III, *thrA* aspartokinase I/homoserine dehydrogenase I, *metL* aspartokinase II/homoserine dehydrogenase II, *asd* aspartate-semialdehyde dehydrogenase, *dapA* dihydrodipicolinate synthetase, *dapB* dihydrodipicolinate reductase, *dapD* tetrahydrodipicolinate succinylase, *dapC* succinyldiaminopimelate aminotransferase, *dapE* succinyl-L-diaminopimelate desuccinylase, *dapF* diaminopimelate epimerase, *lysA* diaminopimelate decarboxylase, *ldcC*/*cadA* lysine decarboxylase, *cadB* diaminopentane-lysine antiporter, *puuA* glutamate-putrescine/glutamate-diaminopentane ligase, *ygjG* putrescine/diaminopentane aminotransferase, *speE* putrescine/diaminopentane aminopropyltransferase and *speG* spermidine acetyltransferase.

In addition to *E. coli*, *C. glutamicum*, which is used for the annual production of 1.5 million tons of L-lysine (Becker and Wittmann 2011), the direct precursor of diaminopentane, appears to be a promising production organism for diaminopentane. However, diaminopentane is not a natural product of the *C. glutamicum* metabolism. Therefore, the

heterologous expression of a lysine decarboxylase gene displays the key modification for the construction of a diaminopentane-producing *C. glutamicum* strain.

Since *C. glutamicum* is the dominating organism for lysine production for many decades, there is substantial knowledge on the pathways of lysine biosynthesis in this organism. Lysine, and therefore also diaminopentane, is synthesized from the carbon building blocks oxaloacetate and pyruvate and requires in addition four moles of NADPH per mol lysine as cofactor (Michal 1999). Thus, diaminopentane biosynthesis is directly connected to the central metabolism via the requirement of building blocks and the reducing power. This central catabolic network of *C. glutamicum* comprises the pathways of glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle and glyoxylate cycle, while the Entner-Douderoff pathway has not been detected (Eikmanns 2005, Kalinowski et al. 2003, Yokota and Lindley 2005) (Figure 3-3). Glycolysis, TCA cycle and anaplerosis are important for the supply of the precursor pyruvate and oxaloacetate, respectively. The required NADPH is produced via glucose 6-phosphate dehydrogenase (Ihnen and Demain 1969, Moritz et al. 2000) and 6-phosphogluconate dehydrogenase (Moritz et al. 2000, Ohnishi et al. 2005), forming the oxidative part of the pentose phosphate pathway, via isocitrate dehydrogenase in the TCA cycle (Eikmanns et al. 1995), and via the gluconeogenic malic enzyme (Gourdon et al. 2000). For interconversion of C_4 metabolites of the TCA cycle and C_3 metabolites of the glycolysis, *C. glutamicum* possesses an extensive set of enzymes. This part of the metabolism plays an important role in the biosynthesis of amino acids of the aspartate family (Sauer and Eikmanns 2005). Pyruvate-carboxylase (Peters-Wendisch et al. 1998) and phosphoenolpyruvate carboxylase (Eikmanns et al. 1989) catalyze the anaplerotic replenishment of the TCA cycle and thus of oxaloacetate. The decarboxylating enzymes, channeling carbon from TCA cycle towards glycolysis, are malic enzyme (Gourdon et al. 2000), phosphoenolpyruvate carboxykinase (Jetten and Sinskey 1993, Riedel et al. 2001), oxaloacetate decarboxylase (Jetten and Sinskey 1995) and probably phosphoenolpyruvate synthetase (Jetten et al. 1994), of which the gene was not yet found.

In a first proof of principle, a *C. glutamicum* wild type was engineered by replacing homoserine dehydrogenase with heterologous lysine decarboxylase (*cadA*) from *E. coli* (Mimitsuka et al. 2007). Whereas this strain still produced lysine at a significant level, diaminopentane was secreted only in small quantities. Production was recently increased via plasmid-based expression of *cadA* using a strong constitutive promoter and kanamycin as selection pressure (Tateno et al. 2009). The yield of diaminopentane only using expression of lysine decarboxylase was, however, still rather low, meaning that this modification can only be a first step towards a competitive industrial strain.

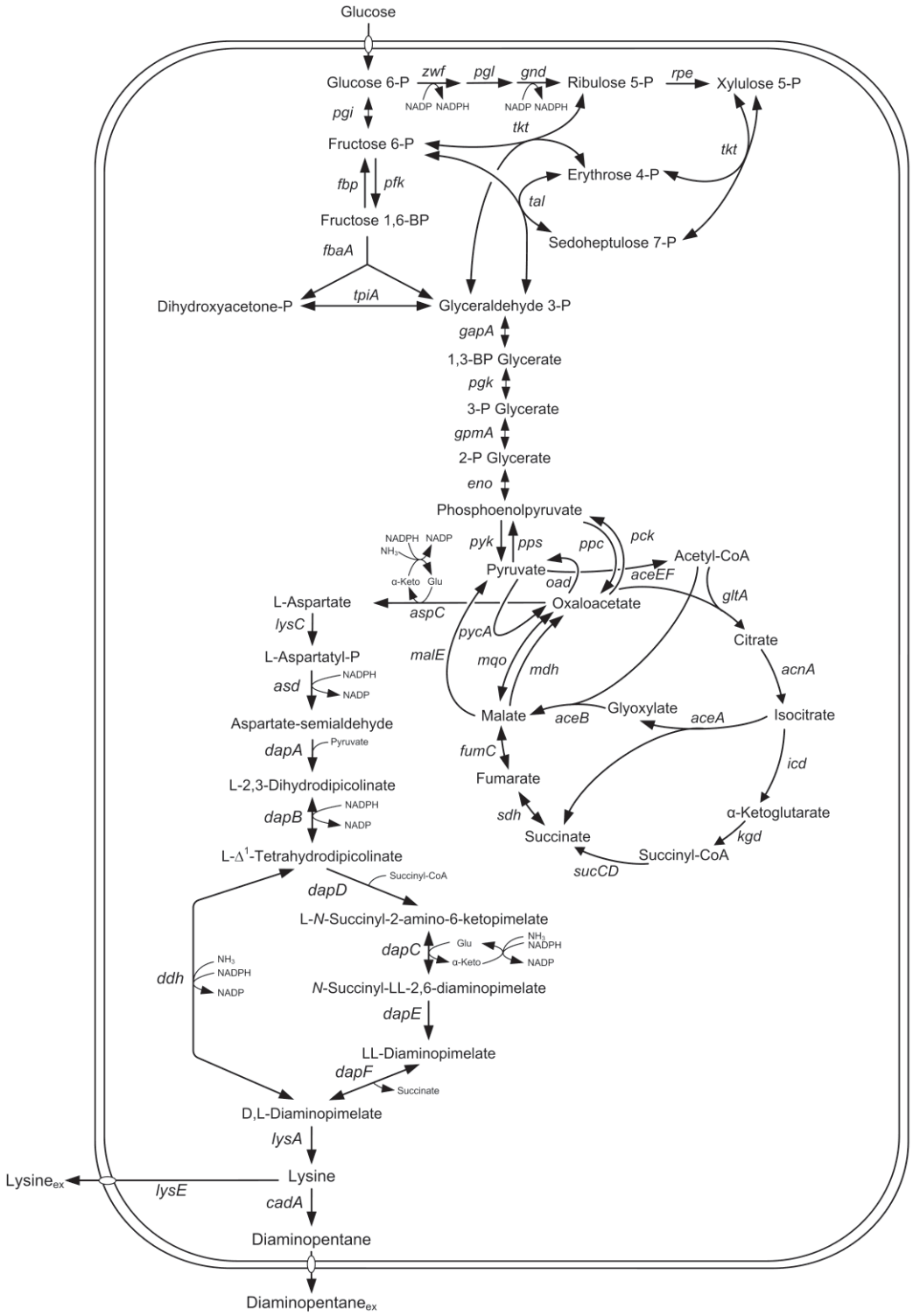

Figure 3-3: Central carbon metabolism of *Corynebacterium glutamicum* and biosynthetic lysine/diaminopentane route via the parallel dehydrogenase and succinylase pathway. The enzymes, involved in diaminopentane formation, encoded by the genes are *lysC* aspartate kinase, *asd* aspartate-semialdehyde dehydrogenase, *dapA* dihydrodipicolinate synthase, *dapB* dihydrodipicolinate reductase and subsequent split into (i) the dehydrogenase pathway with *ddh* diaminopimelate dehydrogenase and (ii) the succinylase-pathway comprising *dapD* tetrahydrodipicolinate succinylase, *dapC* succinyl-amino-ketopimelate transaminase, *dapE* succinyl-diaminopimelate desuccinylase, *dapF* diaminopimelate epimerase. The final common step is catalyzed by *lysA* diaminopimelate decarboxylase. To convert lysine, which is secreted via *lysE* lysine permease, to diaminopentane, *cadA* lysine decarboxylase (from *E. coli*) has to be heterologously expressed.

3.1.3 Bioprocess considerations

The large-scale bio-production using the two most promising diaminopentane producers, *C. glutamicum* and *E. coli*, was well studied lately. This experience has delivered profound knowledge on suitable industrial carbon sources, media supplements, sterilization procedures, and the operational window with regard to fermenter volume, cell density, sugar concentration, pH, or aeration as exemplified for the production of glutamate (Kimura 2005), lysine (Kelle et al. 2005), and tryptophan (Ikeda 2005). This valuable knowledge can be transferred to obtain basic settings for an industrial process, which requires careful coordination of strain characteristics, medium design, and process conditions to ensure a high production performance with minimal variations. Beyond that, product-specific considerations have to be included to optimize such processes. Compared to the traditional production of amino acids as food or feed additives (Stevens and Binder 2000), the downstream processing requirements for diaminopentane as building block for bio-based polymers are different. Pure monomers (>99 %) display a basic prerequisite to ensure an effective polymerization, meaning that the downstream processing has to meet high demands to enable the usage of diaminopentane from microbial fermentation. In order to realize the isolation of secreted diaminopentane from fermentation broth on an industrial scale, a purification process was recently proposed (Völkert et al. 2009). As depicted in the flow diagram in Figure 3-4, the isolation consists of several successive steps. First, cellular components are removed e.g. by separation, flocculation, or filtration processes. Subsequently, the pH is adjusted to alkaline conditions (pH >12) by adding hydroxides of sodium, potassium, or calcium, respectively. Thermal treatment of the fermentation broth, connected to high energy costs, is facultative. Such a step can be included to hydrolyze N-acetyl-diaminopentane, potentially occurring as by-product, and thereby to increase the product titer. The following extraction is carried out with an organic solvent, which is stable at alkaline pH, such as n-butanol. After phase separation, diaminopentane is isolated and purified from the diaminopentane-comprising phase by chromatography, distillation, or precipitation with suitable organic acids. During chromatographic reprocessing, the diaminopentane phase is applied to an acidic ion exchanger. Adding dicarboxylic acids like succinic or sebacic acid for precipitation results in a salt that can be used, either directly or after purification, in a subsequent poly-condensation to receive the appropriate polyamide.

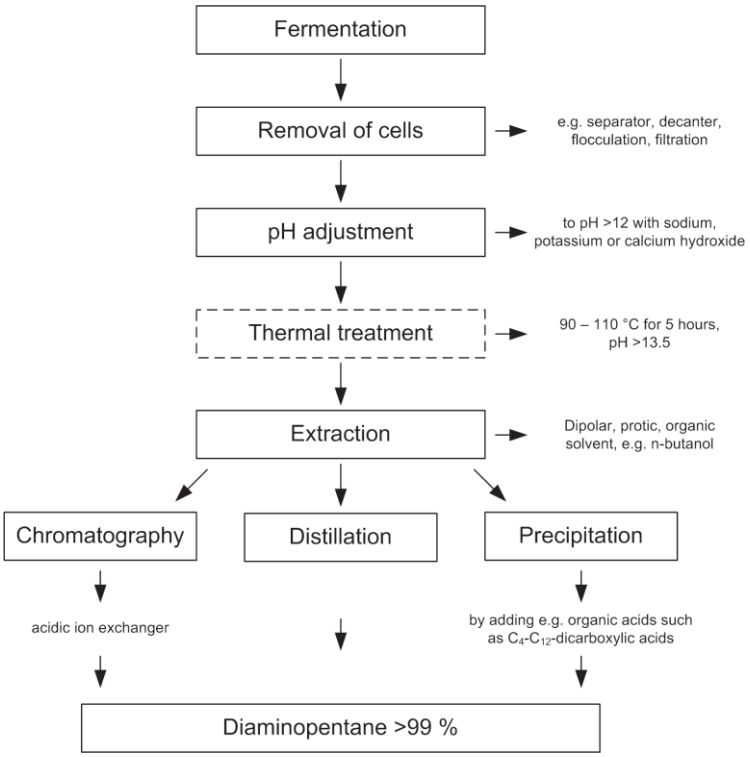

Figure 3-4: Flow diagram of a process for the isolation of diaminopentane from fermentation broth. The dashed box indicates a facultative step.

3.1.4 From diaminopentane to innovative polymers

Today, polyamides comprise various successful materials such as the mass products polyamide 6 or polyamide 6.6 (nylon 6,6) as well as different high-performance polyamides of altered compositions. Together they share an annual global market size of about 6 million tons. Therefore, the production of new polyamides can address an enormous market with product values between 1.3 and 3.5 € per kilogram, and even higher prices for special-polymers, depending on the application (Werpy et al. 2004). The properties of polyamides are largely depending on the basic monomers of which the polyamides are built up. By polymerization of diaminopentane, derived from microbial biosynthesis, with appropriate bio-blocks such as bio-succinate (Guettler et al. 1999, Hong and Lee 2001, Nghiem et al. 1997, Oh et al. 2009, Okino et al. 2008, Stellmacher et al. 2010) or sebacic acid from castor oil (Ogunniyi 2006), completely bio-based polyamides such as PA 5.4 or PA 5.10 are provided (Figure 3-5). Sebacic acid is thereby obtained by extracting or pressing the seed of the castor oil plant, followed by saponification and pyrolysis (Ogunniyi 2006).

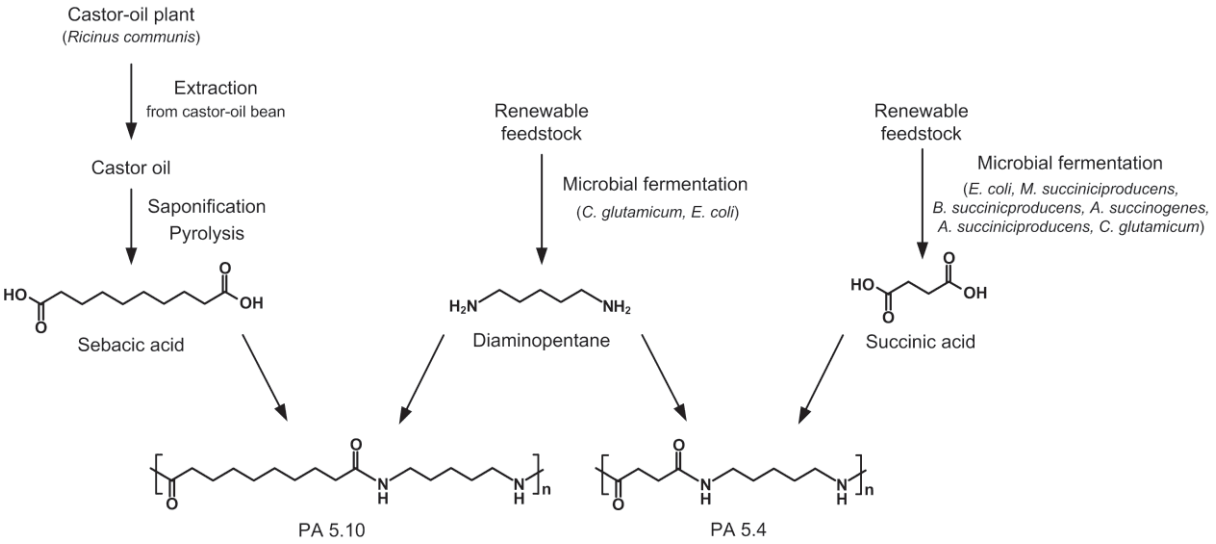

Figure 3-5: Polymerization of diaminopentane with succinic acid or sebacic acid to PA 5.4 or PA 5.10. The use of these monomer building blocks received from renewable resources by microbial fermentation and extraction, respectively, enables the synthesis of completely bio-based polyamides.

The excellent material properties of diaminopentane-based polyamides, as shown by PA 5.10, comprise e.g. a high melting point of 215 °C, which is similar to that of PA 6 (Table 3-1). In some other important characteristics, including density and water absorption, it performs even better than the conventional, petroleum-based nylon, one of the most common industrial polymers. The lower water uptake is advantageous because moisture absorption leads to a decrease in the glass transition temperature and also negatively affects some mechanical properties like E-modulus. Besides, the lower water absorption provides a better long-term dimensional stability, attractive in cases where exact geometrical shapes of the polymer have to be maintained. The lower density, causing a weight saving of about 6 %, is relevant for the use in energy friendly transportation. Other advantages comprise improved flow characteristics and a better visual surface quality (Thielen 2010). Thus, polyamides from diaminopentane open new opportunities for bio-based polymers with advanced material properties for example in the automotive industry or in high-value consumer products. Since, in this field, the currently used compounds are still based on petrochemistry, the advanced material properties of diaminopentane-based polyamides seem especially important concerning a subsequent industrial application.

Table 3-1: Material properties of petroleum-based polyamide PA 6 and bio-based polyamide PA 5.10.

	PA 6	PA 5.10
Bio-based content	0	100
Melting point [°C]	220	215
Glass transition temperature [°C]	54	50
Density [g/cm^3]	1.14	1.07
Water absorption [%]	3.0	1.8

The given data were taken from current literature (Thielen 2010). Melting point and glass transition temperature were determined according to Deutsches Institut für Normung, 1994, and density and water absorption according to Deutsches Institut für Normung, 1998.

3.2 *Corynebacterium glutamicum* as Industrial Workhorse

Corynebacterium glutamicum was first isolated from a soil sample of the Ueno Zoo in Tokyo (Japan) during a research program started in 1956 at Kyowa Hakko Kogyo Co. (Tokyo, Japan), aiming at the discovery of a microorganism that could accumulate glutamate extracellularly (Kinoshita et al. 1957, Kinoshita 2005, Udaka 1960). The isolated microorganism, primarly named *Micrococcus glutamicus*, accumulates glutamate under biotin limiting conditions (Shiio et al. 1962). It belongs to the class *Actinobacteria* and thereunder to the order *Actinomycetales*, the suborder *Corynebacterineae* and the family *Corynebacteriaceae* (Liebl 2005, Stackebrandt et al. 1997). In contrast to pathogen species like *Corynebacterium diphteriae*, *Mycobacterium tuberculosis* and *Mycobacterium leprae*, also belonging to the in nature widely distributed genus *Corynebacterium*, *C. glutamicum* was classified as a GRAS (generally recognized as safe) organism. The non-sporulating, non-motile Gram-positive bacterium with a high GC content of 53.8 % (Kalinowski et al. 2003, Liebl 2005) is characterized by an irregular, rod-shaped to club-shaped morphology during growth (Abe et al. 1967) (Figure 3-6).

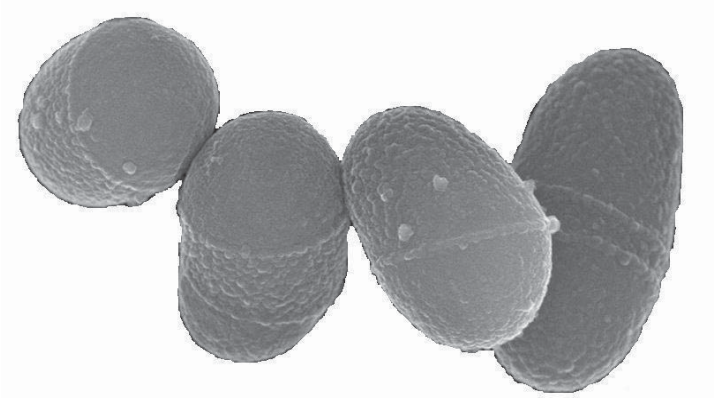

Figure 3-6: Raster electron micrograph of *Corynebacterium glutamicum* during growth in glucose minimal medium (Bolten 2010).

As a GRAS organism, *C. glutamicum* displays an ideal host not only for the production of amino acids, applied as food and feed additives, but also for the production of chemicals and materials like diamines and dicarboxylic acids, and of ethanol and higher alcohols. Glutamate and lysine, with a world market size of 2 and 1.5 million tons per year, respectively, are the major industrial products of *C. glutamicum* (Becker and Wittmann 2011). Besides glutamate and lysine, the L-amino acids threonine (Eikmanns et al. 1991), methionine (Park et al. 2007b), valine (Radmacher et al. 2002), alanine (Jojima et al. 2010), serine (Peters-Wendisch et al. 2005), and histidine (Mizukami et al. 1994) as well as the aromatic amino acids phenylalanine, tyrosine, and tryptophan (Ikeda 2006) are also produced fermentatively using engineered *C. glutamicum*. Beyond the production of amino acids, *C. glutamicum* has been successfully engineered towards the production of the dicarboxylic acid succinate (Inui et al. 2004b), the diamine putrescine (Schneider and Wendisch 2010), and of bio-based fuels like ethanol (Inui et al. 2004a) and isobutanol (Smith et al. 2010). In recent years, an impressive progress can be observed with regard to the biotechnological use of *C. glutamicum*. This progress was made possible by unraveling the genome sequence (Haberhauer et al. 2004, Kalinowski et al. 2003, Tauch et al. 2002), together with the availability of powerful omics technologies for in-depth analysis of *C. glutamicum* and the possibility of the implementation of targeted genetic modifications (Jäger et al. 1992).

3.3 Metabolic Engineering of Diaminopentane-producing *C. glutamicum*

Metabolic engineering is the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones by using recombinant DNA technology (Stephanopoulos 1998). In the past, metabolic pathway manipulation made use of chemical mutagens followed by subsequent selection of superior strains with increased production of a desired metabolite. However, as mutagenesis displays a random process, undesirable modifications can also accumulate during the optimization process. In contrast, metabolic engineering allows a directed modification of specific enzymatic reactions, connected e.g. to product biosynthesis or precursor supply. Besides the decoding of the whole genome sequence, the availability of tools to implement targeted genetic modifications displays a crucial prerequisite for rational strain engineering. For *C. glutamicum*, genetic engineering techniques like plasmid construction and transformation, which are harnessed to amplify, deregulate, delete, or transfer the corresponding gene, have been established over the years (Jäger et al. 1992, Kirchner and Tauch 2003, Tauch et al. 2003, Xie and Tsong 1990). Genetic engineering, applied in an iterative manner, is a

powerful method to systematically improve the production properties of the cell and thereby create a well-defined genetic background, comprising only beneficial modifications.

3.3.1 Diaminopentane biosynthesis and export

As diaminopentane is not a natural product of *C. glutamicum*, implementation of a heterologous lysine decarboxylase, catalyzing the conversion of lysine to diaminopentane, displays a prerequisite for diaminopentane production with this organism. In a pioneering study, the *C. glutamicum* wild type was modified by replacing homoserine dehydrogenase with the lysine decarboxylase gene *cadA* from *E. coli* (Mimitsuka et al. 2007). The obtained strain secreted diaminopentane, even if in relatively small amounts, providing a valuable proof of concept for the production of this novel metabolite in *C. glutamicum*. In a continuative study, the production could be increased via stronger expression of *cadA* using a plasmid-based expression system in combination with a strong constitutive promoter and kanamycin as selection pressure in a homoserine dehydrogenase deletion strain (Tateno et al. 2009). As the yield was with 76 mmol diaminopentane per mol glucose still rather low and the recombinant strains still produced lysine, the expression of lysine decarboxylase can only be regarded as a first step in the construction of a competitive industrial strain.
Superior production strains typically require substantial modification at different levels of the metabolism, including optimization of precursor or cofactor supply within the central carbon metabolism, product export or release from undesired pathway regulation (Park and Lee 2008). In this regard, it seems beneficial that diaminopentane is derived directly from lysine, meaning that previous metabolic engineering strategies of *C. glutamicum* for lysine production seem relevant and helpful (Ohnishi et al. 2002, Sahm et al. 2000, Wittmann and Becker 2007). Within the diaminopentane biosynthetic pathway, which is consistent with the lysine biosynthetic pathway except for the last additional step catalyzed by lysine decarboxylase (Figure 3-3), several targets essential for a high yield are well known. The key enzyme in biosynthesis is, due to its importance in pathway regulation, aspartokinase, which is subject to feedback inhibition by lysine and threonine (Kalinowski et al. 1991, Malumbres and Martin 1996). Different point mutations in the coding region of the regulatory β-subunit as well as overexpression of *lysC* led to improved lysine formation (Cremer et al. 1991, Kalinowski et al. 1991, Ohnishi et al. 2002). Beside *lysC*, almost every enzyme of the biosynthetic pathway was overexpressed and the corresponding mutant was analyzed for enhanced pathway flux. Thereby, overexpression of *dapA* (Cremer et al. 1991, Eggeling et al. 1998), *dapC* and *dapF* (Hartmann et al. 2003) resulted in an improved lysine yield, whereas overexpression of *asd*, *dapB*, *ddh* and *lysA* (Cremer et al. 1991, Eggeling et al. 1998) did not show a positive effect in the tested strain background.

In both diaminopentane-producing *C. glutamicum* strains reported so far, diaminopentane is secreted into the production medium. Based on the charged nature of the product and previous findings for other compounds, an active transport system appears very likely. However, this transport system has not been identified to date.

3.3.2 Central carbon metabolism

In addition to the optimization of the diaminopentane biosynthesis itself, an adequate supply of precursors and cofactors has to be ensured. The availability of oxaloacetate, the direct precursor of the amino acids of the aspartate family, and therefore also of lysine and diaminopentane, is part of a complex network of carboxylating and decarboxylating reactions linked to oxaloacetate supply and withdrawal (Petersen et al. 2000). Overexpression and modification of the major anaplerotic enzyme pyruvate carboxylase have proven beneficial for lysine production (Ohnishi et al. 2002, Peters-Wendisch et al. 2001). Another strategy, involving the pyruvate node, concentrated on the counteracting enzyme PEP carboxykinase. Deletion of this enzyme resulted in an increased anabolic net flux from glycolytic C_3 metabolites to C_4 metabolites of the TCA cycle and in a significantly improved lysine production (Petersen et al. 2001, Riedel et al. 2001).

Furthermore, the down-regulation or deletion of the biosynthetic pathways towards threonine, withdrawing the common precursor aspartate-semialdehyde, has proven useful for lysine production (Ohnishi et al. 2002, Shiio and Sano 1969). In addition, the TCA cycle competes with the lysine/diaminopentane biosynthesis for the precursor oxaloacetate (Melzer et al. 2009, Wittmann and Heinzle 2002). Whereas deletion of the pyruvate dehydrogenase complex introduced negative growth characteristics in addition to an improved lysine production (Blombach et al. 2007), targeted down-regulation of the pyruvate dehydrogenase and isocitrate dehydrogenase gene by start codon exchange, a more moderate genetic modification, caused only positive effects regarding the production properties (Becker et al. 2009, Becker et al. 2010).

Beyond, the pentose phosphate pathway was identified as major source for NADPH in *C. glutamicum* by systems wide metabolic flux analysis (Marx et al. 1996, Wittmann and Heinzle 2002). In this light, successful studies focused on a redirection of the flux through the pentose phosphate pathway in order to increase lysine production. This was realized by direct modification of the NADPH generating enzymes phosphogluconate dehydrogenase (Ohnishi et al. 2005) and glucose 6-phosphate dehydrogenase (Becker et al. 2007). Deletion of the phosphoglucoisomerase, inactivating the glycolytic flux and forcing the metabolization of glucose via the PPP, led to an enhanced lysine formation on this substrate (Marx et al. 2003). Amplified expression of the gluconeogenic enzyme fructose 1,6-bisphosphatase,

resulting in an enhanced PPP flux, was shown to improve the lysine yield on different sugars including glucose, fructose and sucrose (Becker et al. 2005). In another strain background, however, *fbp* overexpression was only beneficial for lysine production on sucrose by significantly decreasing the intracellular concentration of fructose 1,6-bisphosphate, an inhibitor of the key enzymes of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Georgi et al. 2005).

3.3.3 Alternative substrates

For profitable biotechnological production processes, substrates derived from industrial wastes like molasses or starch hydrolyzates, based on glucose, fructose, and sucrose as major carbon sources, are highly relevant substrates. The preceding hydrolysis of starchy materials from corn, wheat, potato, or cassava, necessary since *C. glutamicum* lacks the enzymes involved in starch degradation, is comparatively easy employing mainly α-amylase and glucoamylase enzymes. Therefore, technologies using starchy feedstocks are well-established and highly efficient (McAloon et al. 2000). In addition, heterologous expression of the cell surface attached α-amylase from *Streptococcus bovis* 148 or of the secreted α-amylase from *Streptomyces griseus* IMRU 3570 allowed the production of lysine and diaminopentane directly from soluble starch (Seibold et al. 2006, Tateno et al. 2007, Tateno et al. 2009). As starch displays a food-competing raw material and hence has an equal value for human nutrition, its usage as raw material for the production of chemicals and fuels is controversially discussed (Schneider et al. 2011). Non-food substrates like cellulose and hemicellulose derived from lignocellulosic biomass, ensuring an abundant and cost-effective supply, show future promise (Aristidou and Penttila 2000). The ideal scenario in a biorefinery is a simultaneous saccharification of cellulose and hemicelluloses and co-fermentation carried out by genetically engineered microorganisms that ferment the provided substrates in the same broth as the enzymatic hydrolysis (Mosier et al. 2005). Thereby, the ability of the fermentation organism to utilize a large number of sugars is of major importance. *C. glutamicum* can grow aerobically on a variety of sugars such as glucose, fructose, sucrose, ribose, mannose, or maltose as sole carbon and energy sources (Dominguez et al. 1998, Eikmanns 2005, Gerstmeir et al. 2003, Kiefer et al. 2002, Sasaki et al. 2010, Wittmann et al. 2004a). To broaden the substrate spectrum towards sugars and other carbon sources arising as waste from agriculture or wood industry, *C. glutamicum* has been genetically engineered. Growth on glycerol, available at large amounts as major by-product from the biodiesel industry, was enabled by heterologous, plasmid-based expression of the *E. coli* utilization genes *glpF, glpK* and *glpD* (Rittmann et al. 2008). The major fraction of hemicellulose, derived from lignocellulosic biomass, consists of the pentoses xylose and

arabinose. To a smaller extent, hexoses such as glucose, mannose, or galactose are additionally contained. Implementation of the *E. coli xyl* operon, consisting of xylose isomerase (*xylA*) and xylulokinase (*xylB*), mediated growth of *C. glutamicum* on xylose (Kawaguchi et al. 2006). By expression of the *E. coli araABD* gene cluster, growth and production of organic acids and amino acids from arabinose was possible (Kawaguchi et al. 2008, Schneider et al. 2011). After engineering towards utilization of lactose and galactose by heterologous expression of the lactose and galactose operon from *Lactobacillus delbrueckii* ssp. *bulgaris* and *Lactococcus lactis* ssp. *cremoris*, *C. glutamicum* produced lysine on a whey-based medium (Barrett et al. 2004).

3.3.4 Systems metabolic engineering

Traditional strain optimization involved random mutagenesis, carried out by subjecting the genetic material to a variety of physical or chemical agents, and subsequent screening processes (Parekh et al. 2000). This approach often leads to the accumulation of detrimental mutations, causing e.g. growth deficiency, nutrient auxotrophy or weak stress tolerance. Such problems can be overcome by analysis and targeted modification of the metabolic and regulatory network, using metabolic engineering (Bailey 1991). Based on systems biology, providing sophisticated experimental and computational tools, metabolic engineering and especially systems metabolic engineering allow to overcome the limitations of classical strain engineering and enable the targeted multidimensional alteration of complex networks. Particularly, systems metabolic engineering has recently lifted strain development up to a truly global level and appears most promising for creating a superior production strain for diaminopentane (Becker and Wittmann 2012). This concept implies systems-wide strain design using genome-scale or large-scale stoichiometric modeling (Kjeldsen and Nielsen 2009, Lee et al. 2005, Melzer et al. 2009) for the identification of targets valuable for strain optimization. Omics profiling on the various levels of the cellular network – techniques that are now at hand for *C. glutamicum* thanks to its high industrial relevance – provide an excellent knowledge base for strain optimization. Today, *C. glutamicum* can be analyzed by genome-scale technologies such as genomics, transcriptomics, proteomics, metabolomics, and fluxomics (Becker and Wittmann 2011). Applied in an iterative manner, the cycle of design, implementation and analysis displays a powerful strategy for optimizing diaminopentane production in *C. glutamicum* (Figure 3-7).

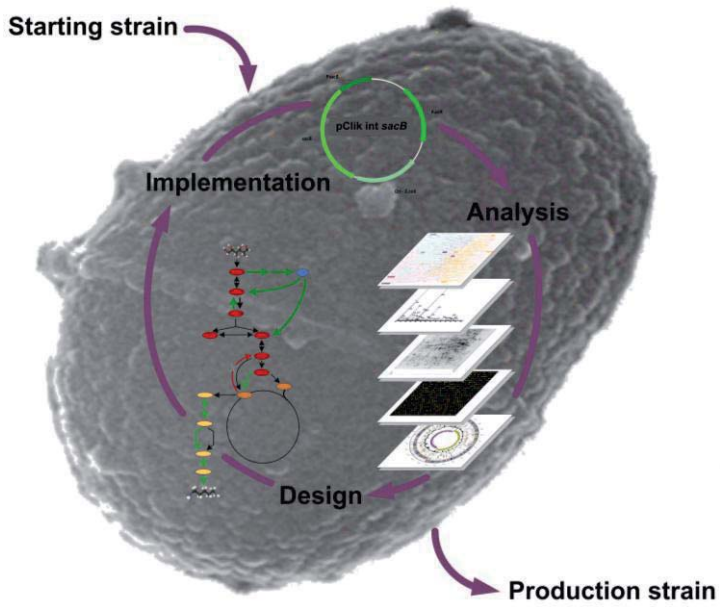

Figure 3-7: Concept for systems metabolic engineering of *C. glutamicum*. The iterative strategy consists of strain design by using *in silico* tools, implementation of the identified target and analysis of the impact of the implemented modification on strain physiology. The scheme for the different omics technologies during analysis was adapted from Kohlstedt et al., 2010.

By comparison of genomes among different organisms or between wild type and mutant strains, genomics allows the identification of genes to be newly introduced, amplified, modified or deleted in order to receive a desired phenotype. This approach was successfully demonstrated for the identification of targets for lysine overproduction (Ikeda et al. 2006, Ohnishi et al. 2005) as well as for the discovery of aminotransferases participating in the biosynthesis of branched-chain amino acids and phenylalanine in *C. glutamicum* (McHardy et al. 2003).

Transcriptome analysis, based on DNA microarrays, provides a global view on gene expression under different conditions or in different strains and thus enables the analysis of cell physiology and global regulations at a systems-wide level (Wendisch 2003, 2006) as well as the identification of potential targets for strain improvement. Further development of this technology towards custom-made microarrays nowadays allows design and construction of arrays perfectly matching the specific requirements. Beside a proper array design, the sampling protocol and the RNA extraction protocol have to be developed for the chosen organism and should be followed by a quality control step to check the integrity of the isolated RNA. For *C. glutamicum*, transcriptome profiling was previously used to get an insight into the gene expression under growth on different carbon sources like glucose and acetate (Gerstmeir et al. 2004, Hayashi et al. 2002, Muffler et al. 2002), during lysine production (Hayashi et al. 2006, Krömer et al. 2004) and into the regulation of the sulfur metabolism (Rey et al. 2005). Additionally, transcriptomics was already applied to identify targets for enhancing the production of glutamate on lactate as carbon source (Stansen et al.

2005), of lysine (Hayashi et al. 2006, Sindelar and Wendisch 2007) and of the branched-chain amino acid valine (Lange et al. 2003).

Proteome analysis, based on 2D-gel electrophoresis followed by identification of protein spots by mass spectrometry, was also established for *C. glutamicum* (Bendt et al. 2003, Hermann et al. 1998, Hermann et al. 2001, Schaffer and Burkovski 2005, Schluesener et al. 2005). As an example of use, proteomics were employed to study the response of *C. glutamicum* to nitrogen limitation (Schmid et al. 2000, Silberbach et al. 2005).

The general aim of metabolome analysis is the identification and quantification of intracellular low-molecular weight metabolites (Börner et al. 2007, Oldiges et al. 2007). Especially in metabolomics, sampling has to be very fast and, because of potential problems connected to sampling, an appropriate protocol is essential (Bolten et al. 2007, Bolten and Wittmann 2008, Wittmann et al. 2004c). In *C. glutamicum*, this technique has proven to be valuable for the investigation of metabolites of the methionine biosynthesis (Krömer et al. 2005, Krömer et al. 2006a, Krömer et al. 2006b) and for the generation of a metabolome profile during lysine biosynthesis (Krömer et al. 2004).

^{13}C metabolic flux analysis, the state-of-the-art technology for the estimation of *in vivo* fluxes of central metabolic pathways, includes an experimental part with the tracer study and the labeling analysis, e.g. via GC-MS, and a computational part with simulation of the labeling data via an isotopomer model (Wittmann and de Graaf 2005, Wittmann 2007). This technique has made an enormous contribution to the understanding and engineering of the metabolism of *C. glutamicum* (Wittmann 2010). Important studies focused on the comparative analysis of fluxes during growth, glutamate and lysine production (Marx et al. 1997, Sonntag et al. 1995), during co-utilization of acetate and glucose (Wendisch et al. 2000), in different genetically engineered *C. glutamicum* strains (Becker et al. 2005, Becker et al. 2007, Becker et al. 2008, Wittmann and Heinzle 2002) and during lysine-production on different carbon sources (Kiefer et al. 2004, Wittmann et al. 2004a).

By integration of data received from all omics studies, providing information at different hierarchical levels of cellular activities, a full description of the physiological state can be achieved. First successful examples of combining two or more omics studies for the analysis and engineering of the cellular metabolism revealed the great potential of such systems-oriented studies in *C. glutamicum*. These studies dealt e.g. with the profiling and physiological characterization of lysine, valine and panthotenate production strains (Hüser et al. 2005, Krömer et al. 2004, Lange et al. 2003). Beside the experimental setup, the visualization of omics data is another key aspect of systems biology, important for both, the analysis and the understanding of the generated data. Today, users can choose from many methods and tools that allow the integration of the available data into a holistic model (Droste et al. 2011, Gehlenborg et al. 2010, Neuweger et al. 2009).