Metabolic engineering of *Corynebacterium glutamicum* for L-lysine production on silage

Dissertation

zur Erlangung des Grades

des Doktors der Naturwissenschaften

der Naturwissenschaftlich-Technischen Fakultät III

Chemie, Pharmazie, Bio- und Werkstoffwissenschaften

der Universität des Saarlandes

von

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Saarbrücken

2012

Tag des Kolloquiums:	18.12.2012
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Men love to wonder, and that is the seed of science.

Ralph Waldo Emerson

Für meine Eltern, Daisy und Lulu

Danksagung

Mein besonderer Dank gilt Herrn Professor Dr. Elmar Heinzle für die Überlassung des Themas, das mir entgegengebrachte Vertrauen und die sehr gute wissenschaftliche Betreuung meiner Arbeit.

Herrn Professor Dr. Gert-Wieland Kohring danke ich für die Bereitschaft zur Begutachtung dieser Arbeit.

Großer Dank gilt auch der Fachagentur für Nachwachsende Rohstoffe (FNR) für die finanzielle Unterstützung, sowie Herrn Professor Dr. Roland Ulber für die Koordination des Projektes.

Sina Balbier, Ines Wagner, Sabrina Lange, Marcel Lambert und Thomas Böse danke ich für ihren experimentellen Beitrag, den sie während ihrer Forschungs-, Bachelor-, Master- und Diplomarbeiten geleistet haben. Für die Unterstützung bei der Analytik danke ich besonders Michel Fritz. Mein Dank geht auch an Veronika Witte, Dr. Klaus Hollemeyer und Robert Schmidt. Ein herzliches Dankeschön geht an Susanne Kohring für ihr unermüdliches Engagement in der Arbeitsgruppe.

Allen Mitarbeitern des Instituts für Technische Biochemie möchte ich für ihre große Hilfsbereitschaft, die schöne Zeit und die einzigartige Atmosphäre danken. Dr. Konstantin Schneider danke ich sehr für seine Unterstützung und die tolle Zusammenarbeit. Ein besonders herzlicher Dank gilt Susanne Peifer, für die sehr hilfreichen Diskussionen und Anregungen sowie für das Teilen der „Doktoranden-Suite“. Vasileios Delis, Tobias Klein, Sabrina Schmeer und Meike Höfner, sowie Verena Schütz und Yongbo Yuan danke ich für die schöne gemeinsame Zeit in der aus Arbeitskollegen Freunde geworden sind.

Meinen Freunden Nanu, Alin, Muhammed, Jimmy und Abdul: vielen Dank! Dr. Thomas Pleli danke ich für seine Unterstützung und Motivation!

Mein besonderer Dank gilt meinen Eltern für ihre unendliche Unterstützung in allen Lebenslagen und dafür, dass sie immer an mich geglaubt haben. Daisy und Lulu danke ich für die schöne Zeit außerhalb des Labors.

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List of symbols and abbreviations

Symbols

c	Concentration	[mol L^{-1}]
μ	Specific growth rate	[h^{-1}]
q_s	Specific uptake rate	[mmol g^{-1} h^{-1}]
q_p	Specific product production rate	[mmol g^{-1} h^{-1}]
T	Temperature	[°C]
t	Time	[h] or [min]
U	Unit	[μmol min^{-1}]
Yx/s	Biomass yield	[g g^{-1}]
Yp/s	Product yield	[g g^{-1}]
OUR	Oxygen uptake rate	[mmol L^{-1} h^{-1}]
CPR	Carbon dioxide production rate	[mmol L^{-1} h^{-1}]
RQ	Respiratory quotient	
DO	Dissolved oxygen	[%]

Abbreviations

2-OXO	2-Oxoglutarate
3PG	3-Phosphoglycerate
ABU	α-Aminobutyrate
AcCoA	Acetyl-Conezyme A
ADP	Adenosine diphosphate
Ala	Alanine

AMP	Adenosine monophosphate
ATCC	American type culture collection
ATP	Adenosine triphosphate
BHI	Brain heart infusion
Bp	base pairs
BSA	Bovine serum albumin
CDW	Cell dry weight
Cit	Citrate
CM	Complex medium
dld	gene encoding D-lactate dehydrogenase
DM	Dry Mass
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E4P	Erythrose 4-phosphate
F6P	Fructose 6-phosphate
F1,6P	Fructose 1,6-bisphosphate
FAD^+	Flavine adenine dinucleotide oxidized form
FADH	Flavine adenine dinucleotide reduced form
fbp	gene encoding fructose 1,6-bisphosphatase
G6P	Glucose 6-phosphate
G6PDH	Glucose 6-phosphate dehydrogenase
GAP	Glyceraldehyde 3-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gapX	gene encoding glycerinealdehyde 3-phosphate dehydrogenase
Glu	Glucose
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate

HPLC	High pressure liquid chromatography
Kan	Kanamycin
Kan^R	Kanamycin resistance
Lac	Lactate
LB	Luria Bertani
ldhA	Gene encoding NAD^+ dependent lactate dehydrogenase
lldD	Gene encoding L-lactate dehydrogenase
LYS	Lysine
lysC	Gene encoding aspartokinase
$lysC^{fbr}$	Feedback resistant aspartokinase
malE	Malic enzyme
MQ	Menaquinone
NAD(P)	Nicotinamide adenine dinucleotide (phosphate) oxidized form
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) reduced form
NH_3	Ammonia
OAA	Oxaloacetate
OD	Optical density
ORI	Origin of replication
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PPP	Pentose phosphate pathway
P_{sod}	Promoter of superoxide dismutase
PTS	Phosphotransferase system
Pyc	Gene encoding pyruvate carboxylase
PYR	Pyruvate
R5P	Ribose 5-phosphate
Rpm	Rounds per minute

RT	Room temperature
S7P	Sedoheptulose 7-phosphate
sacB	Gene encoding levansucrase of *Bacillus subtilis*
SOC	Super optimized broth
sod	Gene encoding superoxide dismutase
SUC	Succinate
Taq	DNA polymerase from *Thermus aquaticus*
TCA	Tricarboxylic acid
Tet	Tetracycline
tkt	Gene encoding transketolase operon
XYL	Xylose

Abstract

Currently the essential amino acid L-lysine is mostly produced using traditional substrates like glucose and molasses. In this work, a computer based approach and molecular biology techniques were applied in order to investigate the potential of L-lysine overproduction with *C. glutamicum* on renewable substrates, in this case silage and silage juice. Based on elementary mode analysis, several target genes in the feedback resistant *C. glutamicum* $lysC^{fbr}$ strain, including D-lactate dehydrogenase (*dld*), pyruvate carboxylase (*pyc*), malic enzyme (*malE*), fructose 1,6-bisphosphatase (*fbp*) and glyceraldehyde 3-phosphate dehydrogenase (*gapX*), were overexpressed. Substantially re–designing the metabolism yielded mutants with good growth characteristics, complete substrate consumption, reduced byproduct formation coupled with increasing L-lysine yields on synthetic and natural silage juices. This combination of mutations, beneficial for the use of gluconeogenic substrates and sufficient anabolic reduction power, yielded a robust and stable strain, *C. glutamicum SL*. With a total L-lysine carbon yield of around 10% at growth rates of $\mu = 0.35 \pm 0.01$ h^{-1} on grass and corn silage juices with no further supplementation and hardly affected by low oxygen supply, this strain proves the suitability of bio-based L-lysine production.

Zusammenfassung

Für die Produktion der essentiellen Aminosäure L-Lysin wurden bisher traditionelle Kohlenstoffquellen wie Glukose und Molasse benutzt. Unter Zuhilfenahme bioinformatischer und molekularbiologischer Methoden wurde die Einsatzmöglichkeit von Silage und Silagepresssaft als Fermentationssubstrat für eine Lysinproduktion mit *C. glutamicum* geprüft. Basierend auf der durchgeführten Elementarmodenanalyse wurden im Ausgangsstamm *C. glutamicum lysC*^{fbr} die D-Laktat Dehydrogenase (*dld*), Pyruvatcarboxylase (*pyc*), Malatenzym (*malE*), Fruktose-1,6–bisphosphatase (*fbp*) und die Glycerinaldehyd-3-phosphat-Dehydrogenase (*gapX*) überexprimiert. Die Umstrukturierung des Zentralstoffwechsels lieferte Mutanten mit guten Wachstumsraten, einem breiteren Substratspektrum, geringerer Nebenproduktbildung und erhöhten Lysinausbeuten auf synthetischen und natürlichen Silagepresssäften. Durch die Kombination an Mutationen, die eine bessere Verwertung von glukoneogenen Substraten und eine ausreichende NADPH Versorgung ermöglicht, entstand der robuste und stabile Stamm *C. glutamicum SL*. Mit Lysinausbeuten von 10% bei einer Wachstumsrate $\mu = 0.35 \pm 0.01$ h^{-1} auf verschiedenen Silagepresssäften auch bei reduzierter Sauerstoffversorgung, zeigt dieser Stamm ein großes Potential für Lysinproduktion auf Silagen als nachwachsendem Rohstoff.

Scope and outline of the thesis

The focus of this thesis is on metabolic engineering of L-lysine production with *C. glutamicum* using silage and silage juice as substrate. Contrary to commonly used substrates, silage juice contains high amounts of lactate and other organic acids, requiring smart engineering in order to satisfy the demand of carbon building blocks and redox power needed for L-lysine production. While *C. glutamicum* wild type is not able to grow on silage juice, elementary mode analysis provided useful targets for genetic engineering of the metabolism. Once applied, growth and L-lysine production steadily improved. The utilization of the various carbon sources was investigated in detail, linking their consumption to strain specific parameters like growth rate and L-lysine production. This work has the potential to pave the way for a larger spectrum of biotechnological products, evaluating silage as a renewable, sustainable substrate for biotechnological production.

In **Chapter 1** we provide a general introduction on the necessity of replacing commonly used petrochemical feedstock with bio-based feedstock for the production of chemicals, in this case the amino acid L-lysine.

The best candidate for this application, *C. glutamicum*, is introduced in **Chapter 2** as a production platform for various chemicals, especially amino acids. Computational methods and bioinformatics combined with targeted genetic modifications provide a very effective toolbox for target identification and metabolic engineering of this organism.

The design and performance of created mutants on synthetic silage juice is described in detail in **Chapter 3**, showing the improved substrate spectrum of the created mutants, the improved growth behavior and L-lysine production.

In **Chapter 4** we focus on different silages and methods of processing as well as drawbacks related to formation of inhibitory compounds during the treatment of the feedstock. The performance of engineered mutants on different silage juices and under different cultivation conditions is described in detail.

Pretreatment methods for corn silage juice and potential applications are described in **Chapter 5**.

In **Chapter 6** we summarize the findings presented in **Chapters 3-5** and discuss their potential use and contribution to a sustainable, bio-based economy along with an outlook on future research in this field.

Chapter 1

General introduction

General introduction

With over 7 billion people and the numbers still growing, the efficient use of our natural resources is one of the top challenges of our time (Pinstrup-Andersen and Pandya-Lorch, 1998). Economic growth is greatly depending on safe, sustainable resources for industrial production of chemicals and transportation fuels. Currently, only a minor fraction of the chemical industries output is based on renewable raw materials (Lichtenthaler and Peters, 2004). For the major part, the chemical industry relies on fossil resources, coupled with environmentally damaging production processes, waste and very bad eco-efficiency (Mecking, 2004). The constant search for oil and the subsequent exploitation of these fossil resources have negative effects on environment and society. The constantly increasing global demand for petroleum based goods will soon exceed the exploitation, resulting in a peaking of oil prices and the depletion of these resources in near future (Bentley, 2002). Pollution, immense costs for waste disposal and a rapid rise in the costs of mineral oil are increasing the pressure to make chemical production more eco-friendly. For the environment, non-degradable or non-recyclable products as well as toxic byproducts represent a serious ecological challenge (Wackernagel and Yount, 2000). Furthermore, the emission of carbon dioxide and other greenhouse gases in the atmosphere and the related global warming calls for attention (Yu et al., 2008). All these facts are pointing in the same direction, a shift from fossil to bio-based raw materials. An attractive alternative feedstock for the production of chemicals and fuels is biomass. Green biomass, including agricultural and forestry residues, energy crops, grasses and woody plants being one of the largest sources of renewable, sustainable resources worldwide (Clark, 2007; Hall and Scrase, 1998; Hoffert et al., 1998), can be used to produce biogas and liquid fuels to generate electricity or to create bio-based products like plastics, resins and fine chemicals. The production of plant biomass is vast, sufficient to match the demand of food and feeds with the demand for the production of chemicals and fuels (Dale, 2003; Ericsson and Nilsson, 2006). Optimal use of agricultural and forestry residues, waste biomass and the cultivation of dedicated crops for non food production is a vantage point for the bio-based industry (Hill, 2000). Breeding to increase the yield performance of plants and even alter the cellular composition of these raw materials is already practiced (Boehmel et al., 2008). One of the major benefits of using bio-based chemicals instead of petroleum based ones is the closed carbon cycle. While storage of CO_2 in fossil raw materials has taken millions of years, the CO_2 generated by the combustion of bio-based fuels can be incorporated in

biomass via photosynthesis in a much shorter period of time (van Maris et al., 2006). Bio-based chemicals can be biodegraded into CO_2 and water which are both used during photosynthesis for the regeneration of biomass (Figure 1.1).

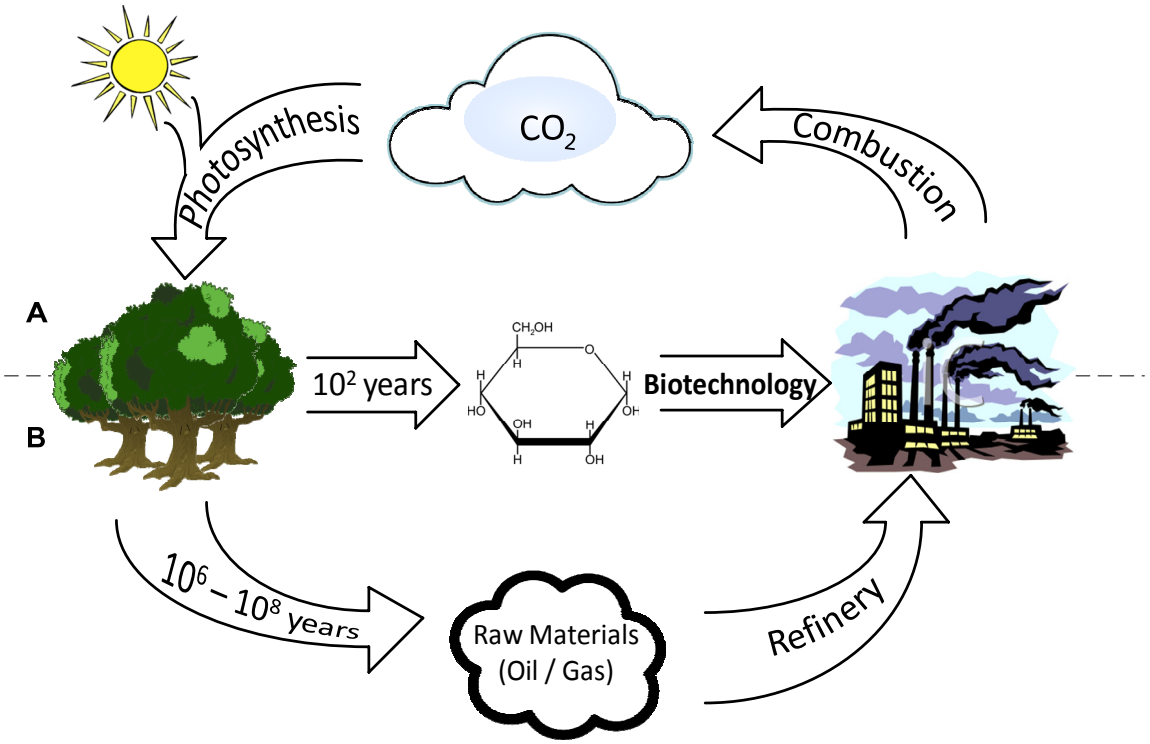


Figure 1.1: Schematic overview of the carbon cycle for A: the biotechnological route, or B: the petrochemical route. Adapted from van Maris et al. (2006).

But a change in the feedstock can only occur, if the technological basis for its industrial processing is altered. Linking the production of chemicals and biotechnological processes in general with the rapidly emerging bio energy industries is a promising starting point (Kamm and Kamm, 2004; Kamm and Kamm, 2007). Analogous to the petroleum refinery, the concept of the biorefinery has emerged (Figure 1.2). The processing facilities of a biorefinery are centered on agricultural or farming units, where biomass is converted into various compounds that are fed into various product lines.

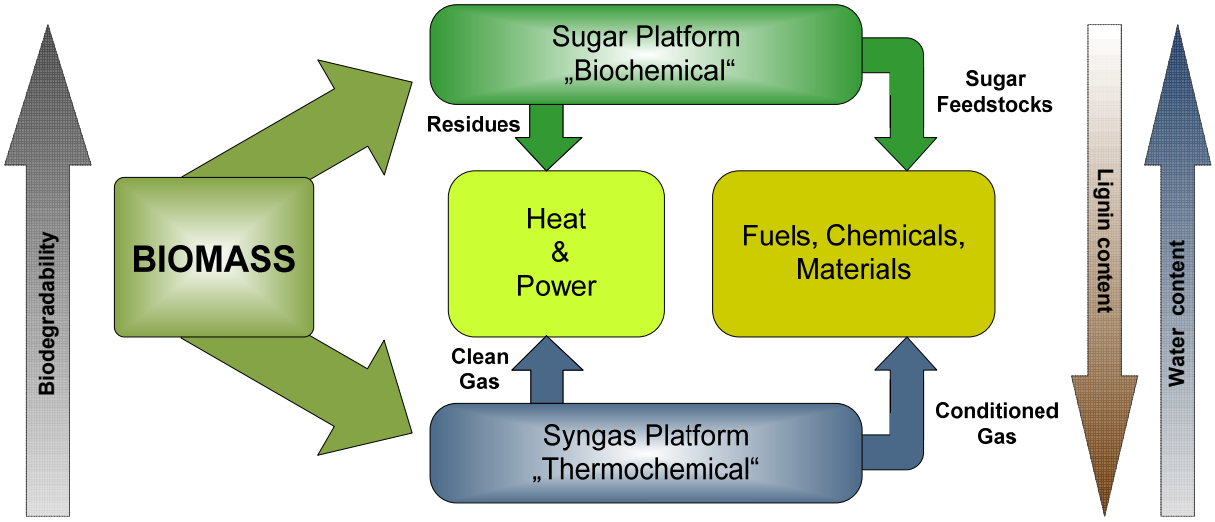


Figure 1.2: Schematic concept of a biorefinery. Based on http://www.nrel.gov/biomass/biorefinery.html.

The natural complexity and divergency of biomass can be useful for the production of multiple products, maximizing the value derived from biomass. Compared to conventional production, in a long run, sustainable production systems should be more profitable, since they use materials and energy more efficiently reducing also the associated waste production. This can be achieved, by producing bio-based products that are durable, less toxic, recyclable and biodegradable, while performing well, compared to their conventional counterparts. An industry is truly sustainable when it is economically viable, environmentally compatible and socially responsible.

Role of biotechnology in sustainability

One of the core tools facilitating the shift from fossil to bio-based production is biotechnology. Also known as white biotechnology, the industrial biotechnology is often able to comply several green chemistry principles. Multi step chemical synthesis can be replaced with a single step synthesis of desired products with a lot less energy and material input as well as reduced waste generation, organic solvent free media, selective catalysis and biodegradable products (Sijbesma and Schepens, 2003). Furthermore, white biotechnology enables the synthesis of products that cannot be synthesized chemically

(Gavrilescu and Chisti, 2005). With a growing number of organisms entering the post genomic era and advances made in the fields of enzyme and metabolic engineering as well as proteomics and bioinformatics, the choice for suitable microorganisms and enzymes for bioconversions is facilitated. Screening for novel organisms, isolated from extreme environments (Madigan and Marrs, 1997) or the use of metagenomics (Lorenz et al., 2002) helps discovering new biocatalysts. *In vitro* evolution of such enzymes can further improve the enzyme activity, achieving much higher conversion rates, than in nature. The combination of high titer, tailor made production strains and engineered enzymes can for example improve biocatalysis for biomass hydrolysis, one of the key components for biorefineries. Biorefineries represent a complex system of ecological technologies for the comprehensive material and energetic utilization of such renewable raw materials, produced in a sustainable way, where useful products are synthesized without producing any new waste (Kamm and Kamm, 2004). Especially in Europe, most green biorefineries use silage instead of fresh, green biomass, guaranteeing a constant substrate supply throughout the year. This ensures a more decentralized system, with the farmers being more integrated by running the silos, providing social sustainability for the whole concept (Hanegraaf et al., 1998).

Traditional biotechnological processes have been used for years. Microbial production of enzymes, antibiotics or amino acids are only a few examples (Hermann and Patel, 2007). Regarding amino acids, the history of microbial amino acid production started in the late 1950's, when Dr. Kinoshita discovered that *C. glutamicum* is a superior amino acid producer. Until then, amino acids were either extracted or produced by chemical synthesis. Today, over 2 million tons of amino acids per year are produced by microbial fermentations using genetically engineered strains, with a market growth of around 10% per year (Leuchtenberger et al., 2005). In the era of bioeconomy, producer strains are no longer engineered only towards a desired product but also regarding the ability to efficiently use alternative, renewable substrates. In this context, we decided to investigate the use of silage and silage juices for the production of L-lysine.

Silage

A more effective way to preserve the biomass and make it available throughout the year is ensiling (McDonald et al., 1991). Ensiling or silaging is the process of preserving plant material in undried and anaerobic conditions, either in a storage silo or wrapped in plastic. It is an old method of moist forage preservation, accounting for more than 200 million tons of biomass stored annually in Europe and the US (Weinberg and Muck, 1996). After the harvest, the green plant material is chopped and left to wither up to a dry mass content of approximatively 30% and finally pressed to remove remaining oxygen and assure anaerobic conditions. Based on natural fermentation, lactic acid bacteria convert a large fraction of the present water-soluble carbohydrates into lactic acid. As a result, the pH decreases, inhibiting detrimental anaerobes. If the forage is too moist, not allowing a sufficient pH decline, clostridia, which ferment lactic acid to butyric acid and amino acids to ammonia, might become active. This process is referred to as "secondary or clostridial fermentation" (Weinberg and Muck, 1996). When anaerobic conditions are not guaranteed, the presence of oxygen enables various aerobic spoilage microorganisms to become active (Woolford, 1990). The most frequently involved microorganisms in aerobic deterioration of silages are yeasts and acetic acid bacteria, followed by bacilli, moulds and enterococci (McDonald et al., 1991; Spoelstra et al., 1988). After providing anaerobic conditions, lactic acid bacteria develop and become the predominant microbial population. The produced lactic acid is an interesting product for the manufacture of biodegradable polymers (Södergard, 2002). The downside of the lactic acid production is the recovery cost (Datta et al., 1995). Instead of extracting lactic acid from the silage juice, we try to use it as an additional carbon source for L-lysine production (Neuner and Heinzle, 2011).

Table 1.1: Different types of silage and their composition.

Plant	Grass	Corn	Sugar Beet
			
Dry mass (DM)	35 – 45%	25 – 30%	30 – 35%
Protein	135 g kg^{-1}	80 g kg^{-1}	110 g kg^{-1}
Lactic acid	~200 g kg^{-1}	~130 g kg^{-1}	~160 g kg^{-1}
WSC* + Starch	~45 g kg^{-1}	~300 g kg^{-1}	~30 g kg^{-1}
Fiber	240 g kg^{-1}	200 g kg^{-1}	210 g kg^{-1}
Potential	5 – 15 DM/ha/a	15 – 20 DM/ha/a	10 – 15 DM/ha/a
Yield	172 m^3/t MM	202 m^3/t MM	67 m^3/t MM
Biogas	54% methane	52% methane	72% methane

* water soluble carbohydrates

On a large scale, silage is either used for the production of biogas (Table 1.1) or as fodder at farming units (Kromus et al., 2004). With meat production stagnating and milk production reaching higher productivities per cow, the percentage of utilized grassland will decrease and grassland will become a surplus problem for agriculture. In the next decade, 50-100 million hectares of land in the EU may become available for purposes other than food crop production (Weiland, 2003). This opens up new substrate opportunities for biotechnical engineering with two conceivable options. The first one would be the integration of L-lysine fermentation into the process of a green biorefinery (Figure 1.3) and the subsequent use of the purified product, L-lysine as a fine chemical.

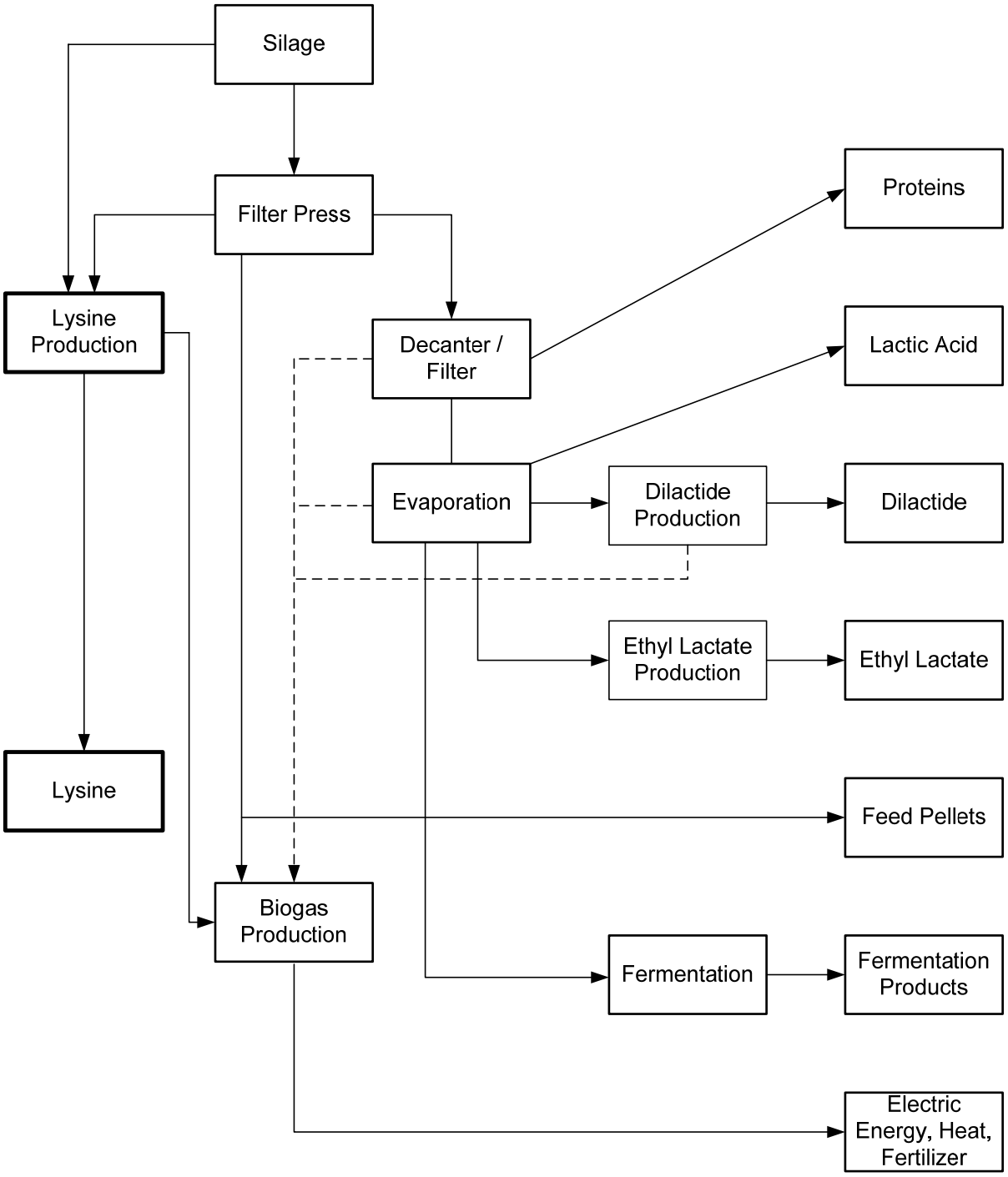


Figure 1.3: The product pipeline of a biorefinery. Adapted from Kromus et al.(2004)

This would be an additional, waste free step in the production pipeline of a biorefinery, contributing to a sustained value enhancement, with all the residues being used for biogas production. Offering considerable environmental benefits and an additional income source for the farmers, biogas production from biomass is of growing importance. The biogas yield from cow and pig manure is low due to two factors, insufficient organic dry matter

content (2-10%) and most of the energy rich substances have been digested by the animals (Amon et al., 2007). This leads to the conclusion, that the use of manure as the sole substrate for biogas production is not economical and makes the use of co-substrates necessary. An evaluation of modern biogas plants showed that corn and grass silage are the most applied co-substrates in agricultural biogas plants. Over 80% of the plants are operated with simultaneous fermentations of manure and silage. Figure 1.4 displays the application frequency of various co-substrates for biogas production (Weiland, 2003).

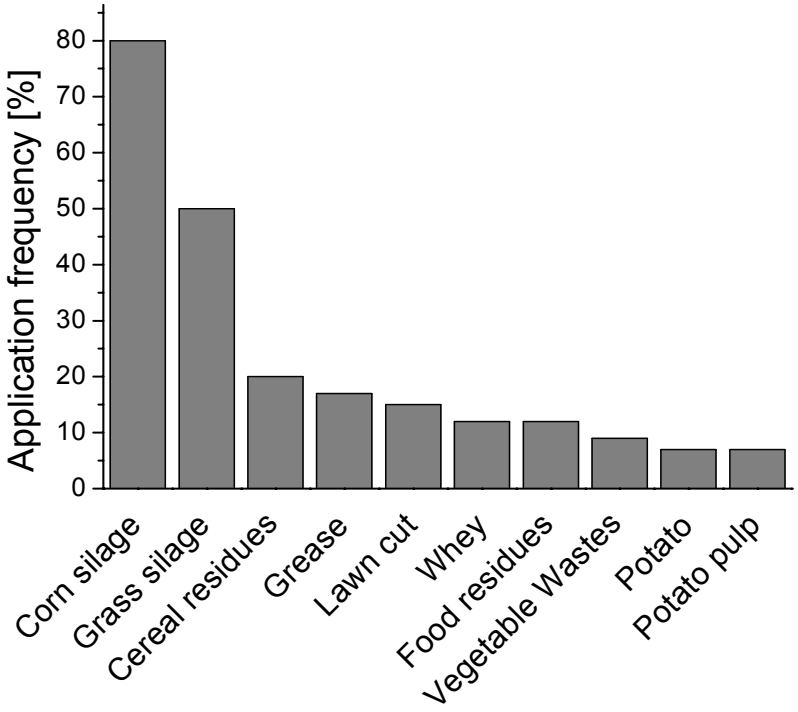


Figure 1.4: Application frequency of various co substrates (Weiland, 2003).

Wastes from food industries, greases and fat containing residues as well as residues from restaurants, markets and the municipal area are mainly used in large, centralized plants, because of the needed pretreatment and the required technological equipment. Silage and byproducts from agricultural industries as well as energy crops are therefore very interesting alternatives as co-substrates and for mono fermentations in smaller farming units, since they do not require sophisticated special treatment before use. Besides that, the fertilizer quality of the digestate is high (Angelidaki et al., 2009).

The second option would be the supplementation of fodder with L-lysine without any product purification. Proteins are essential components in feeds. Many amino acids can be synthesized by the animal itself. In the case of the essential amino acids, the animal relies upon the amino acids supplied by the food. Vegetable feeds have an own characteristic amino acid spectrum, which differs from that of the animal. L-lysine supplementation is dramatically increasing the biological value of most plant proteins, since they tend to be poor regarding L-lysine (Belitz, 2001; Mitchell and Smuts, 1932). This issue is discussed since the 1930's (Mitchell and Smuts, 1932) and it is still unsolved today (Krajcovicova-Kudlackova et al., 2005), mainly because of the high prices for the needed supplementation. In order to increase the feed efficiency, L-lysine rich crops, like soybean meal are added to traditional animal fodder like corn, wheat and barley. An advantage is the increased food quality that is associated with the drawback that other amino acids from soybean meal are not used by the animals and therefore increase the amount of secreted nitrogen significantly. In terms of fodder, one of the most important aspects is the PER (protein efficiency ratio). The PER is based on the weight gain of a test subject divided by its intake of a particular protein (Belitz, 2001). It is an indicator of the protein quality. Maize, which is the best producer of both calories and protein per acre (Johnson and Lay, 1974), exhibits a PER of 0.85. A L-lysine supplementation of 0.4% increases the PER to 1.08. Further addition of 0.07% tryptophan results in a PER of 2.5. In case of wheat protein, a 0.2% L-lysine supplementation is raising the PER from the initial value of 0.65 to 1.56, making L-lysine the most used amino acid in fodder supplementation (Belitz, 2001), improving the animals ability to utilize nitrogen strongly enhancing growth (Leclercq, 1998) and decreases the release of nitrogen into the environment. A carefully adjusted amino acid supplementation allows a reduction in the nitrogen release of about 60%, attributing L-lysine a considerable ecological relevance to the farming industry (Kircher and Pfefferle, 2001). Since *Corynebacterium glutamicum* belongs to the GRAS category of microorganisms, the L-lysine enriched fermentation broth could be added to the fodder without any further purification.

Chapter 2

Corynebacterium glutamicum as a platform for biotechnological production

Corynebacterium glutamicum as a platform for biotechnological production

Corynebacterium glutamicum is a Gram positive, non-motile, aerobic soil bacterium with a GC content of 54.1% (Pfefferle et al., 2003). Like *Arthrobacter* and *Brevibacterium*, it belongs to the group of coryneforme bacteria, named after the typically rod shape with clubbed ends (Figure 2.1). Contrary to the related pathogenic species, *Corynebacterum diphteriae* and *Mycobacterium tuberculosis,* it is non pathogenic, and exhibits the GRAS status (Burkovski, 2008).

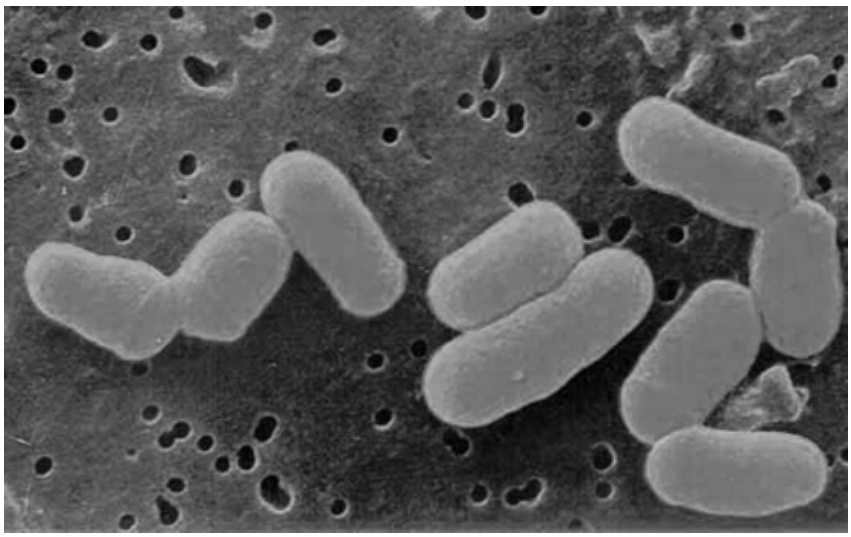


Figure 2.1: Microscopical image of *Corynebacterium glutamicum* (source: whymashen.wordpress.com).

It was originally isolated from a soil sample of the Ueno Zoo in Japan (Udaka, 1960). In the late 1950's, when Dr. Kinoshita started a research program aimed at obtaining a microorganism able to secrete amino acids (Kinoshita et al., 2004), the industrial biotechnology was not aware of the role *Corynebacterium glutamicum* was going to play in the future. Starting as a natural glutamate producer, *C. glutamicum* emerged to one of

the top players in biotechnological production. With a yearly production of over 2 million tons L-glutamate and 1.5 million tons of L-lysine, amino acids are still on top of all biotechnological products of *C. glutamicum* (Kohl and Tauch, 2009). After entering the post genomic era (Tauch et al., 2002a), the variety of products synthesized but also of substrates used by *C. glutamicum* increased dramatically (Becker and Wittmann, 2011). Being naturally able to metabolize various carbon sources, including different sugars and organic acids, genetically engineered *C. glutamicum* strains with extended substrate spectrum were created, e.g. utilizing xylose (Kawaguchi et al., 2006), starch (Seibold et al., 2006; Tateno et al., 2007), arabinose (Schneider et al., 2011), glycerol (Rittmann et al., 2008) and many others. The flexible and robust metabolism combined with advanced omics technologies (Wittmann, 2010), targeted genetic engineering (Jäger et al., 1992) and bioinformatics (Neuner and Heinzle, 2011; Schilling et al., 2000; Schuster et al., 1999) successfully broadened *C. glutamicum's* product portfolio. Besides various amino acids including non proteinogenic amino acids, diamines like cadaverine (Kind et al., 2011; Mimitsuka et al., 2007) and putrescine (Schneider et al., 2012; Schneider and Wendisch, 2010), vitamins, dicarboxylic acids (Okino et al., 2008), polymers (PHB) (Liu et al., 2007), higher alcohols (Smith et al., 2010) and fuels (Inui et al., 2004a) are all products of this versatile key player in industrial biotechnology.

Central carbon metabolism

The central metabolic network of *C. glutamicum* comprises the pathways of glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, the glyoxylate shunt and various anaplerotic and cataplerotic reactions, while the Entner Doudoroff pathway is missing (Eikmanns, 2005; Kalinowski et al., 2003). Regarding L-lysine biosynthesis, the central metabolic pathways provide the precursors oxaloacetate and pyruvate, energy in form of ATP and GTP as well as the redox equivalent NADPH. NADPH is needed as a cofactor for the biosynthesis of different amino acids, including L-lysine, L-methionine and L-tryptophan, exhibiting the highest NADPH demand. Especially concerning L-lysine production, the supply of this cofactor is of special interest, since L-lysine biosynthesis is coupled to a high demand of 4 mol NADPH per mol of L-lysine. A key characteristic of

superior L-lysine producer strains is a sufficient NADPH supply. In *C. glutamicum*, two enzymes of the oxidative branch of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase as well as the TCA located isocitrate dehydrogenase and the malic enzyme are NADPH generating reactions (Gourdon et al., 2000; Marx et al., 1996; Wittmann and de Graaf, 2005). NADPH consuming reactions are besides L-lysine biosynthesis the growth associated anabolic reactions with a associated stoichiometric demand of 16.4 mmol NADPH (g biomass)$^{-1}$ (Wittmann and de Graaf, 2005).

Glycolysis

The glycolysis is important for the supply of precursors for cellular anabolism. Besides that, it provides energy in form of ATP. Hexoses like glucose and fructose are metabolized in the glycolysis and further oxidized in the TCA. In glycolysis, a set of ten reactions is converting 1 mol glucose to 2 mol pyruvate, gaining 2 mol ATP and 2 mol NADH. Two of these ten reactions are irreversible and have to be bypassed when gluconeogenic substrates like lactate and acetate are used (Cocaign-Bousquet and Lindley, 1995; Netzer et al., 2004). These two bypassing reactions are catalyzed by PEP carboxykinase and fructose 1,6-bisphosphatase (Stryer, 2007). Glycolytic and gluconeogenic activity is strictly regulated, depending on the organisms needs and available carbon sources. One of the key regulators in glycolysis/gluconeogenesis is the fructose 1,6-bisphosphatase (Rittmann et al., 2003). Increased amounts of AMP, indicating low energy levels in the cell have inhibitory effects on fructose 1,6-bisphosphatase, downregulating gluconeogenesis and promoting glycolysis (Shiio et al., 1990).

TCA cycle

In addition to supplying essential redox equivalents and energy, the TCA cycle also provides precursors required for biomass formation. Since these intermediates are constantly withdrawn for anabolism, an extensive set of different enzymes is responsible for the anaplerotic replenishment of these metabolites (Petersen et al., 2000). Especially when overproduction of amino acids belonging to the aspartate or glutamate family is

intended and OAA is constantly needed for their biosynthesis, the anaplerotic enzymes and the C_3/C_4 metabolism play a major role (Sauer and Eikmanns, 2005). The enzymes PEP- and pyruvate carboxylase replenish the OAA pool, in order to maintain the functionality of the TCA cycle. The C_4 decarboxylating enzymes PEP-carboxykinase, OAA decarboxylase and the malic enzyme replenish the phosphoenolpyruvate and pyruvate pools, all together forming the so called PEP-pyruvate-oxaloacetate node of *C. glutamicum* (Eikmanns, 2005). This anaplerotic node represents the link between glycolysis and the TCA cycle. At the same time, when gluconeogenic carbon sources like lactate, acetate or a TCA cycle intermediate are used, the anaplerotic node is the starting point of gluconeogenesis.

Pentose phosphate pathway

The general role of the pentose phosphate pathway (PPP) is the supply of anabolic reducing power in form of NADPH and precursor metabolites like ribose 5-phosphate and erythrose 4-phosphate, used for the synthesis of aromatic amino acids and nucleotide- and nucleoside production. It has two branches, the oxidative, irreversible route, where glucose 6-phosphate is converted to ribulose 5-phosphate by three enzymes, with the formation of 2 moles of NADPH per mol glucose (Moritz et al., 2000). The reversible, non oxidative route, including the enzymes transketolase and transaldolase is not generating any redox equivalents. The role of the non oxidative route is the isomerization, epimerization and interconversion of C_3, C_4, C_5, C_6 and C_7 compounds. The PPP is an alternative to glycolysis, with a rather anabolic than catabolic role, branching at glucose 6-phosphate and refueling glycolysis at the levels of fructose 6-phosphate and glyceraldehyde 3-phosphate. Especially in L-lysine producers, with a higher requirement of NADPH, the flux partitioning through the PPP is of major interest (Moritz et al., 2000; Wittmann and Becker, 2007).

Lactate metabolism

While the uptake system for L-lactate has not been unequivocally identified, *C. glutamicum* can grow aerobically on L-lactate as a carbon and energy source (Cocaign-Bousquet and Lindley, 1995; Cocaign et al., 1993; Gourdon et al., 2000; Seletzky et al., 2006). In a first step L-lactate is oxidized to pyruvate via L-lactate dehydrogenase encoded by the *lldD* gene (Bott and Niebisch, 2003; Schluesener et al., 2005; Stansen et al., 2005). The utilization of L-lactate is mainly regulated by the expression levels of *lldD*, which was shown to be organized in an operon with a putative L-lactate permease (NCgl2816). Compared to cells grown on minimal medium containing glucose, fructose or pyruvate, the mRNA levels of this operon were 18-fold higher when L-lactate was used, indicating a carbon source dependent transcriptional regulation (Burkovski, 2008; Stansen et al., 2005). The genome sequence revealed another gene, *dld*. The protein encoded by the *dld* gene shows 46% sequence identity to a D-lactate dehydrogenase from *E. coli* and several other bacteria (Burkovski, 2008). While the *lldD* is inducible, *dld* is constitutively expressed, at very low levels (Scheer et al., 1988). Both dehydrogenases are peripheral membrane proteins, using menaquinone as electron acceptor. *C. glutamicum* possesses a third, soluble, NAD^+ dependent lactate dehydrogenase, encoded by the *ldhA* gene (Stansen et al., 2005). While the NAD^+ dependent lactate dehydrogenase LdhA is used for reoxidation of NADH under conditions, where the respiratory chain is limiting, the menaquinone dependent dehydrogenases Dld and LldD are mainly involved in lactate oxidation, when used as a carbon and energy source (Burkovski, 2008; Eggeling and Bott, 2005). The formed pyruvate is channeled into the TCA cycle by the pyruvate dehydrogenase complex and the pyruvate carboxylase. Lactate utilization has also a major impact on expression levels of the gluconeogenic enzymes PEP carboxykinase and fructose 1,6-bisphosphatase. Both enzymes activity and mRNA concentrations are 5 fold higher in lactate grown cells compared to glucose grown cells. This indicates a transcriptional control of these steps in gluconeogenesis (Burkovski, 2008; Georgi et al., 2008).

L-lysine production and cofactor economy in *C. glutamicum*

L-lysine production in *C. glutamicum* is closely linked to various supporting pathways. For the synthesis of one L-lysine molecule, one molecule of oxaloacetate and pyruvate, 4 molecules of NADPH, two molecules of ATP and one molecule of NH_3 are used.

L-lysine is part of the aspartate family of amino acids, consisting of L-lysine, L-threonine, L-methionine, L-isoleucine and the non proteinogenic amino acid D,L-diaminopimelate, the direct precursor of L-lysine and an important building block for cell wall synthesis (Wehrmann et al., 1998). The steps towards L-lysine biosynthesis (Figure 2.2) include the phosphorylation of aspartate by aspartate kinase and its subsequent reduction to aspartate semialdehyde. After the condensation step catalyzed by dihydrodipicolinate synthase, which converts aspartate semialdehyde to dihydrodipicolinate and its reduction to piperideine dicarboxylate, the pathway may split. Contrary to *E. coli*, where L-lysine is synthesized in four different steps, converting piperideine dicarboxylate to D,L-diaminopimelate, *C. glutamicum* is able to catalyse this conversion in one step by direct ammonium incorporatrion by the diaminopimelate dehydrogenase (Bartlett and White, 1985). In depth analysis revealed that *C. glutamicum* exhibits both variants of D,L-diaminopimelate synthesis, called the succinylase way and the dehydrogenase way, respectively (Schrumpf et al., 1991; Wehrmann et al., 1998). Further studies revealed that flux distributions through the two available routes is mainly dictated by the availability of ammonium (Misono and Soda, 1980). This allows *C. glutamicum* and other bacteria, including *Bacillus macerans*, which posses this split pathway, to flexibly adapt to alterations in the nitrogen supply (Wehrmann et al., 1998) (Sonntag et al., 1993).

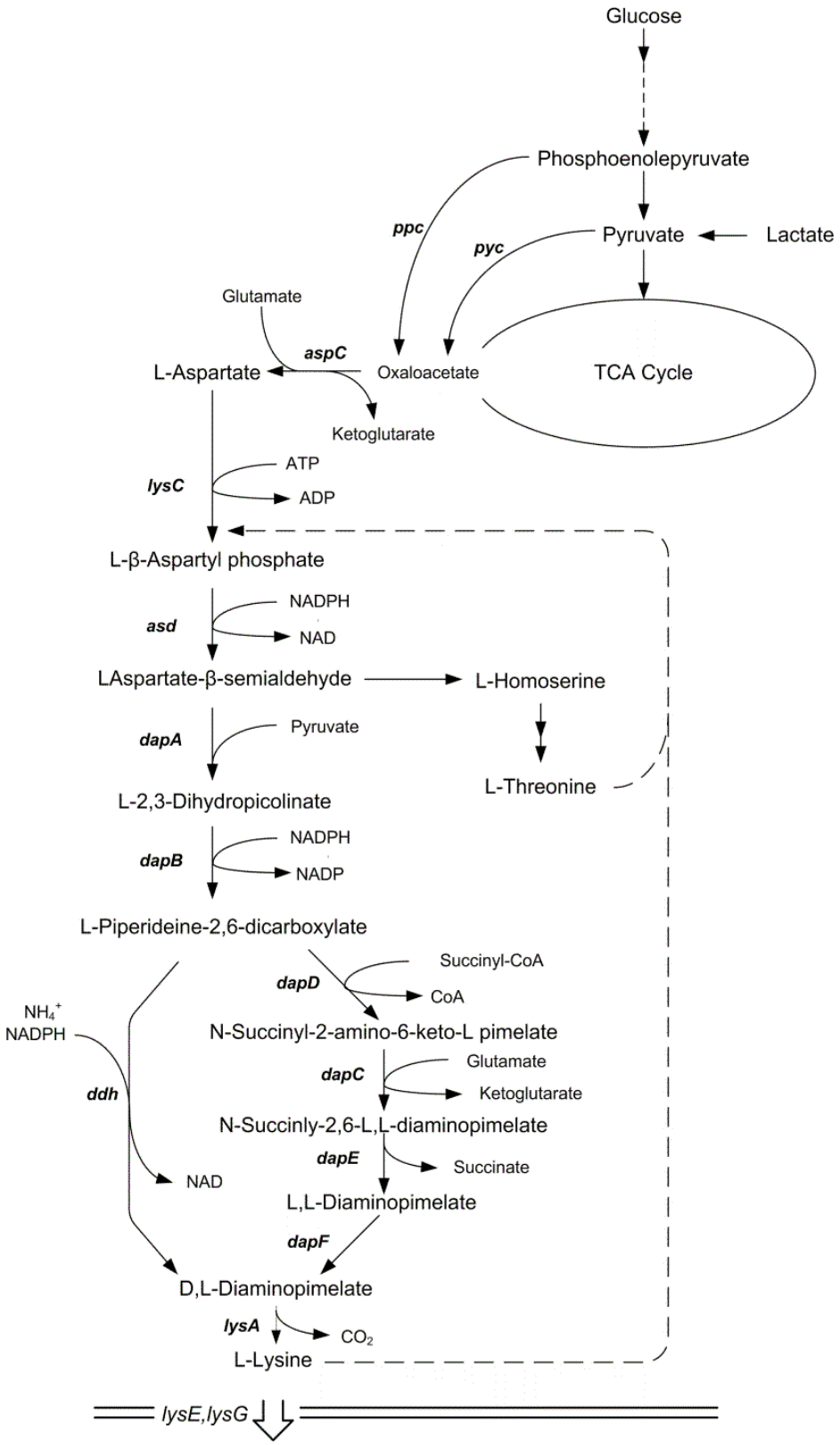


Figure 2.2: Pathway and regulation of L-Lysine biosynthesis in *C. glutamicum*. The enzymes encoded by the genes are *ppc* – phosphoenolpyruvate carboxylase, *pyc* – pyruvate carboxylase, *aspC* – aspartate aminotransferase, *lysC* – aspartokinase, *asd* –

aspartate semialdehyde dehydrogenase, *hom* – homoserine dehydrogenase, *dapA* – dihydrodipicolinate synthetase, *dapB* – dihydrodipicolinate reductase, *dapD* – tetrahydrodipicolinate succinylase, *dapC* – succinyl-diaminopimelate aminotransferase, *dapE* – succinyl-L-diaminopimelate desuccinylase, *dapF* – diaminopimelate epimerase, *lysA* – diaminopimelate decarboxylase, *ddh* – diaminopimelate dehydrogenase, *lysE* – L-lysine exporter, *lysG* – L-lysine export regulator. The broken arrow indicates feedback inhibition.

The synthesis of amino acids is strictly regulated, to cover the organisms demand and avoid unnecessary production. In case of L-lysine, the regulation mechanism is a feedback inhibition of the aspartate kinase by the endproducts of the pathway, L-lysine and L-threonine. By binding at the regulatory β subunit, the affinity of the catalytic α subunit for the substrate aspartate decreases and the biosynthesis is diminished (Stryer, 2007). In order to overproduce L-lysine, the feedback inhibition must be deregulated. Product feedback inhibition of allosteric enzymes is an essential issue for the development of highly efficient microbial strains for bioproduction. Exchange of a single base in the *lysC* gene, encoding the aspartate kinase may lead to feedback resistant strains, e.g. the substitution of threonine with isoleucine at the position 311 in the amino acid sequence of the enyzme (Kim et al., 2006). The result is a deregulated aspartate kinase and a reduction of the feedback inhibition and therefore overproduction of L-lysine. In the last 50 years considerable efforts to deregulate this enzyme from allosteric inhibition by L-lysine and L-threonine have been made, identifying partially or completely desensitized mutants (Chen et al., 2011).

Systems metabolic engineering of *C. glutamicum*

Many industrial strains for the production of useful metabolites have been developed by classical whole cell random mutagenesis. Repeated random mutation and selection yielded producer strains with remarkable characteristic qualities (Demain, 2000; Rowlands, 1984). Although this classical method has enabled great progress in the biotechnological industry, it has serious disadvantages. Regarding industrially important properties like growth and stress tolerance, classically derived production strains are often inferior to the wild type strains and the disadvantage of supplementing the defined medium with essential metabolites due to the auxotrophy increased production costs (Kiss and Stephanopoulos, 1992). Besides the desired mutations, a lot of uncharacterized mutations might occur, some being detrimental to the strains performance. Undefined mutations leave the production mechanism undetermined, an impediment for further rational metabolic engineering. It was obvious that future success in increasing the performance and yield of already highly productive strains will depend on the availability of detailed information on the metabolic pathways and their regulations (Sahm et al., 1995). During the 1980's, several *C. glutamicum* genes from the biosynthetic pathways leading to the aspartate-family amino acids L-lysine, L-threonine, and L-isoleucine, have been cloned and analyzed (Sahm et al., 2000). These studies led to a better understanding of the metabolic pathways, but a complete insight into the complex interactions was impossible, due to the lack of detailed genetic information. However, many successful examples of strain engineering using molecular techniques have been reported (Cremer et al., 1991; Ikeda and Katsumata, 1992; Shiio et al., 1984). A more rational design, based on biochemical and physiological data obtained from continuous cultures was developed. Implementing these data, a mathematical formulation based on balancing extracellular substrate consumption as well as biomass and product formation rates enabled a novel way of analyzing the complex metabolic network of *C. glutamicum* (Stephanopoulos, 1999). Developed and applied by Vallino and Stephanopolous, metabolite balancing was successfully used to determine metabolic fluxes and identify potential bottlenecks (Vallino and Stephanopoulos, 1993). However, this approach has certain limitations due to the required constraints. By entering the post genomic era, the missing information for the rational, engineered development of industrial *C. glutamicum* strains finally became available (Ikeda and Nakagawa, 2003;

Kalinowski et al., 2003; Ohnishi et al., 2002). Together with significant progress in the development of analytical techniques (Wittmann and Heinzle, 1999; Wittmann and Heinzle, 2001a) the application of ^{13}C isotope labeling (Heinzle et al., 2008; Wiechert et al., 2001; Yang et al., 2006) and related isotopomer modeling, the prerequisites for more elaborated metabolic flux analysis, namely an exact cellular composition, existing reactions and network topology were available. A quantitative knowledge about the *in vivo* activity of certain pathways as well as their contribution to the overall activity of the cell has proven crucial for an in depth understanding of *C. glutamicum* and for further optimization of producer strains (Krömer et al., 2004; Sauer and Eikmanns, 2005; Wendisch et al., 2006; Wittmann and Becker, 2007; Wittmann and de Graaf, 2005; Wittmann and Heinzle, 2001b; Wittmann and Heinzle, 2002; Yang et al., 2005). Innovative methods for genetic manipulation (Kirchner and Tauch, 2003; Vertes et al., 2005) combined with powerful bioinformatical tools (Neuner and Heinzle, 2011; Radhakrishnan et al., 2010; Schuster et al., 1999; Schuster et al., 2007; Stelling et al., 2002) for target identification, the overexpression or deletion of genes using recombinant DNA techniques (Kirchner and Tauch, 2003) is today's top method for the design and construction of strains with a desired genotype (Becker et al., 2011; Kind et al., 2010; Krömer et al., 2006; Wittmann and Becker, 2007).

Targeted genetic engineering

Though various studies are still carried out using plasmids for the expression of genes,the targeted genetic engineering of *C. glutamicum* is nowadays primarily performed via homologous recombination (Kirchner and Tauch, 2003). The genetic construct, containing a 500 bp homologous region is synthesized via PCR. The genetic modifications include deletions (Marx et al., 2003; Schwarzer and Puhler, 1991), overexpression (Becker et al., 2005; Neuner and Heinzle, 2011), point mutations (Kim et al., 2006), start codon exchange (Becker et al., 2011) and heterologous gene expression (Liebl et al., 1992). All these genetic modifications and the corresponding genetic constructs are performed via fusion – PCR. The principle of this so called SOE – PCR is described in Figure 2.3.

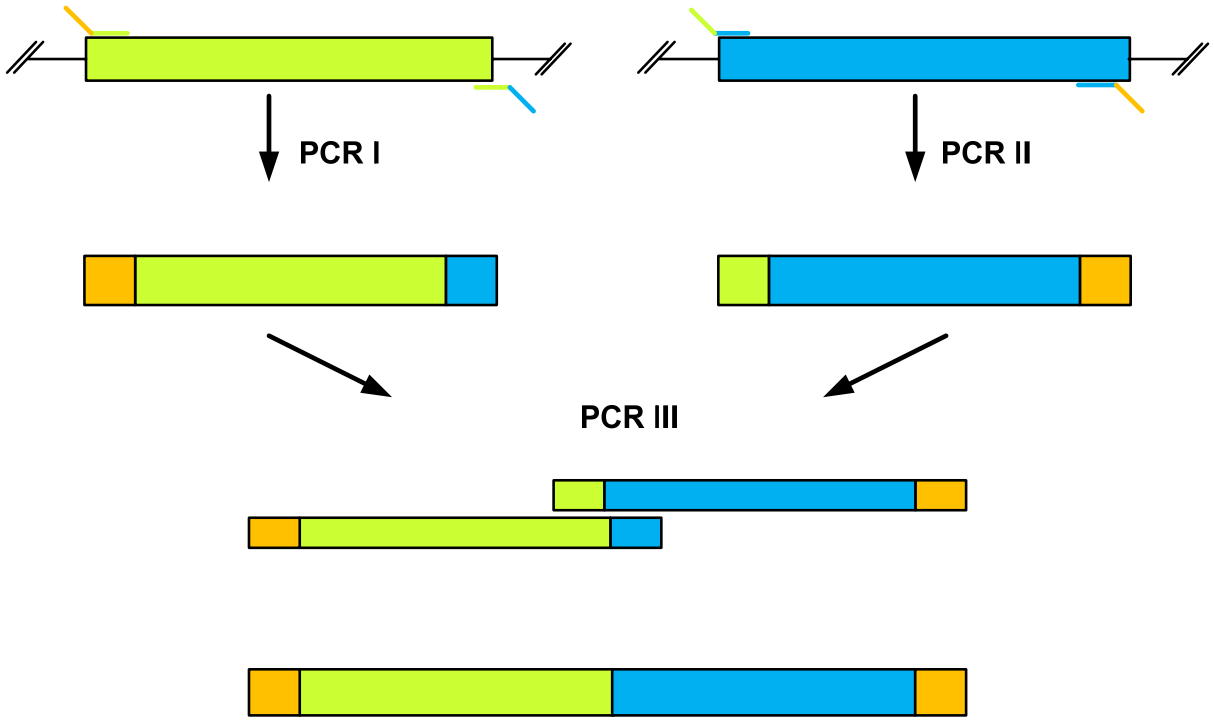


Figure 2.3: Principle of the fusion PCR, connecting two DNA fragments.

The stable integration of the modified DNA into the genome is facilitated by the use of integrative plasmids. These plasmids possess an ORi (*origin of replication*) for *E. coli* but not for *C. glutamicum*. The plasmids containing the engineered insert are amplified in *E. coli* and receive the specific methylation pattern of *C. glutamicum*. The information for the methylation is encoded on the pTC plasmid, contained in the used *E. coli* strain. This methylation step is dramatically increasing the transformation efficiency, since *C. glutamicum* is degrading xenogenic DNA (Bonamy et al., 2003; Tzvetkov et al., 2003). The purified, methylated plasmids are further used for transformation of *C. glutamicum*. The commonly used transformation technique is electroporation (Kirchner and Tauch, 2003; Liebl et al., 1989). Since the integrative plasmids have no ORI for *C. glutamicum*, only cells with an integrated plasmid can replicate and form colonies on selective agar plates. The selection of the 1st recombination occurs via kanamycin resistance. Testing of the clones is performed via PCR, where the positive clones display both alleles, the wild type allele and the engineered one. Positive clones can be directly used for the 2nd recombination. During the 2nd recombination, the wild type allele should be replaced by the engineered one via homologous recombination. Selection of the 2nd recombination

clones is facilitated by the second selection marker *sacB*, encoding for the levan sucrose, an exoenzyme from *Bacillus subtilis*. This enzyme degrades sucrose to levan, a toxic substance for *C. glutamicum* (Jäger et al., 1992). Clones still possessing the pClick int sacB plasmid can express the levane sucrose and are not able to grow on agar plates containing sucrose. Only positive mutants that performed the allelic exchange and wild type cells with the rejected plasmid can grow on sucrose containing plates. The verification of the 2nd recombination is also done by PCR. The engineered DNA section is sequenced, in order to exclude undesired mutations. Validation of the mutant strains is carried out by enzyme tests and cultivation experiments. A schematic overview of the recombination steps for genome based modifications in *C. glutamicum* is depicted in Figure 2.4.

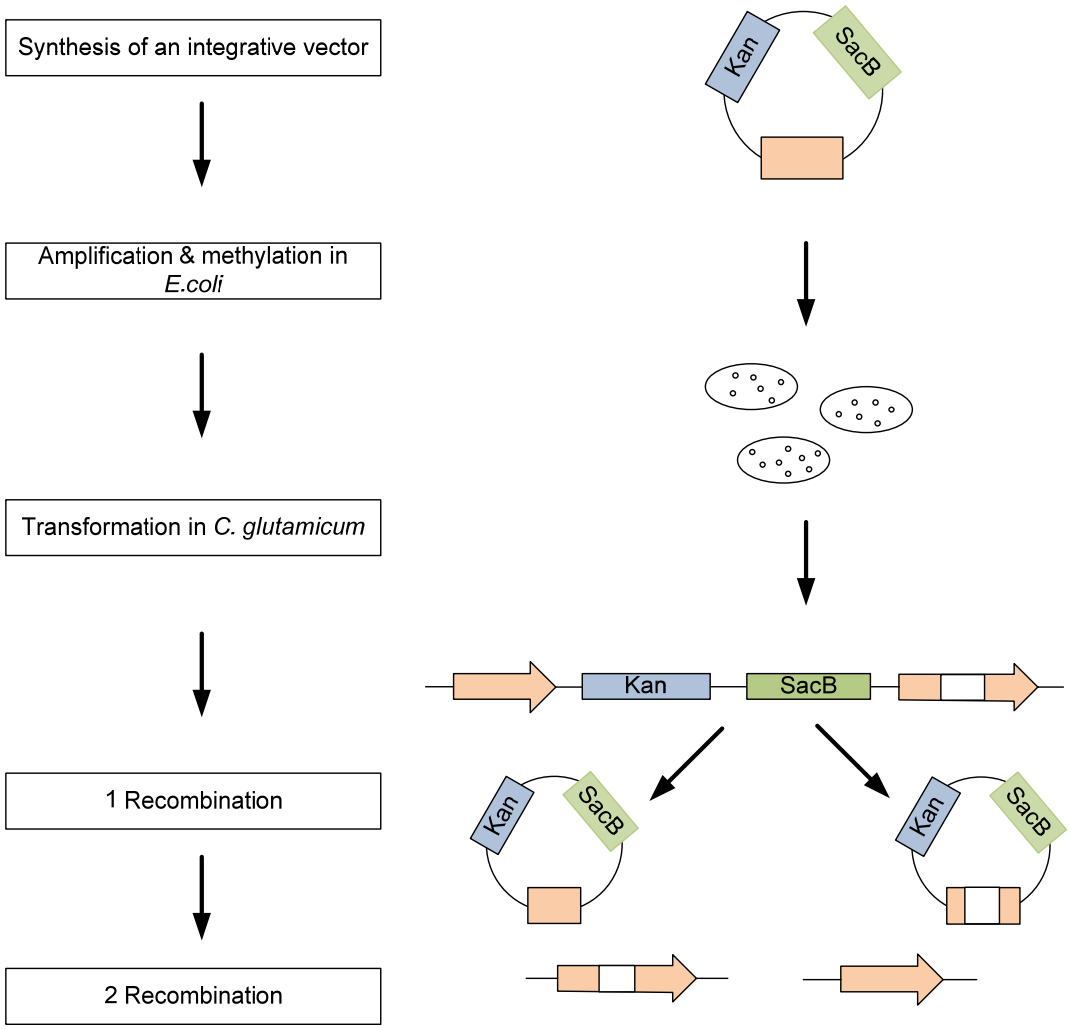


Figure 2.4: Recombination steps for genome based modifications in *C. glutamicum*.

Elementary mode analysis

The engineering of microorganisms for overproduction of desired chemicals involves simultaneous optimization of multiple objectives like productivity, extended substrate range and improved tolerance. The achievement of all these objectives is almost impossible without mathematical modeling and simulations. Different algorithms have been developed, based on a stoichiometric network comprising all educts, intermediate metabolites and products, while kinetic parameters are not explicitly required for the calculations (Papin et al., 2004; Schilling et al., 2000; Schuster et al., 1999; Schuster et al., 2007). Since more advanced sequencing technologies provided gene sequences of many different organisms (Benson et al., 2008; Kanehisa et al., 2008; Karp et al., 2007), metabolic pathway analysis became an irreplaceable component, a basis for rational strain design. This type of analysis has been successfully applied to various organisms in order to investigate their metabolic network structure, robustness or regulation, providing a rigorous basis to systematically characterize certain genotypes and the corresponding phenotypes (Carlson and Srienc, 2004a; Carlson and Srienc, 2004b; Poolman et al., 2004; Price et al., 2002; Price et al., 2003; Wiback et al., 2004; Wlaschin et al., 2006). The elementary mode analysis (EMA) is decomposing metabolic networks of highly interconnected reactions into uniquely organized pathways. Each pathway is consisting of a minimal set of irreversible reactions which operate as a functional unit at steady state, leading from substrate to product. Removal of any reaction in an elementary mode will automatically disrupt the entire pathway. By eliminating inefficient pathways, engineered strains can be forced to function only according to efficient pathways, coupled to cell growth (Pfeiffer et al., 1999; Schuster et al., 1999). Metabolic pathway analysis also allows the determination of the overall capacity, i. e. the maximal theoretical yield, showing potential effects of genetic modifications. This information is very useful, since the economic efficiency and feasibility of the process can be estimated. This also allows the comparison between different organisms (Krömer et al., 2006). The use of silage and silage juices as sustainable, renewable carbon sources instead of the commonly used glucose as a fermentation substrate requires tailored strains, with a broader substrate range. Therefore, instead of the commonly used, product oriented approach, we applied a similar but more advanced, product and substrate oriented approach. We adapted our computational method to the complexity of the substrate, silage. Our focus was on three main carbon sources,

glucose, fructose and lactate. We accounted for possible variations regarding their concentration, depending on the used green biomass, ensiling techniques and seasonal factors like temperature, humidity and harvest time. Detailed information and the results of the applied elementary mode analysis are given in Chapter 3.

Chapter 3

Mixed glucose and lactate uptake by *Corynebacterium glutamicum* through metabolic engineering*

*Published as: **Andreas Neuner** and Elmar Heinzle (2011): Mixed glucose and lactate uptake by *Corynebacterium glutamicum* through metabolic engineering, *Biotechnology Journal*, Volume 6, Issue 3, pages 318-329, DOI:10.1002/biot.201000307

Abstract

C. glutamicum ATCC 13032 *lysC*^{fbr} strain was engineered to grow fast on racemic mixtures of lactate and to secrete L-lysine during growth on lactate as well as on mixtures of lactate and glucose. The wild type *C. glutamicum* grows only well on L-lactate. Overexpression of D-lactate dehydrogenase, *dld*, carried out by exchanging the native promoter of the *dld* gene with the stronger promoter of the *sod* gene encoding superoxide dismutase in *C. glutamicum* resulted in a duplication of biomass yield and faster growth without any secretion of L-lysine. Elementary mode analysis was applied to identify potential targets for L-lysine production from lactate as well as from mixtures of lactate and glucose. Two targets for overexpression were pyruvate carboxylase and malic enzyme. The overexpression of these genes using again the *sod* promoter resulted in growth associated production of L-lysine with lactate as sole carbon source with a carbon yield of 9% and of 15% during growth on a mixture of lactate and glucose. Both substrates were taken up simultaneously with a slight preference for lactate. As surmised from the elementary mode analysis, deletion of glucose-6-phosphate isomerase resulted in a decreased production of L-lysine on the mixed substrate. Elementary mode analysis together with suitable objective functions has been found a very useful guide for the design of strains producing L-lysine on mixed substrates.

Introduction

Raw material supply is a key factor in the production of bulk biochemicals like amino acids. Traditionally waste materials like molasses, various hydrolysates but also starch, e.g. from corn, have been used. In countries with moderate climate with their seasonal changes sustained raw material supply for fermentation is restricted to stabilized materials like starch or molasses. An alternative method of stabilization of agricultural raw materials is silage that has been used for a long time for feed preservation. Silage is extensively and increasingly used in agricultural and cattle industry, since it has a 70% higher nutritional value than hay (Van Soest, 1982). More recently silage is also used for the production of biogas (Kromus et al., 2004) and serves such as a renewable resource for the supply of methane. Two major compounds of silage juice produced from grass are lactate and glucose (Krotscheck et al., 2004). Lactate is considered primarily responsible for conservation of the material, i.e. restriction of the growth of other bacteria. It was now interesting whether such type of raw material could potentially be used to produce more valuable compounds than methane. Potentially useful products are essential amino acids like L-lysine or L-methionine which are extensively used as animal feed additives (Ikeda, 2003). *C. glutamicum*, a gram positive soil bacterium grows aerobically on various carbohydrates and organic acids as carbon sources (Liebl et al., 1991) and is the most frequently used organism for industrial production of amino acids (Wittmann, 2010). Glucose and lactate are co-metabolized (Cocaign et al., 1993). Considerable knowledge about the genes and enzymes involved in glucose and lactate metabolism is available (Gourdon et al., 2000; Stansen et al., 2005). Nevertheless, growth on glucose-lactate mixtures has only been studied marginally and, to our knowledge, no attempt to produce amino acids from lactate and mixtures of lactate and glucose using engineered strains has been reported. *C. glutamicum* has two genes encoding quinone dependent lactate dehydrogenases, L–lactate dehydrogenase (*lldD*) and D–lactate dehydrogenase (*dld*). Both are peripheral membrane proteins (Schluesener et al., 2005), serving to oxidize lactate when it is used as a carbon and energy source. *LldD* is transcribed together with NCgl2816, coding for a putative permease, in one operon. Additional lactate carriers seem to exist in *C. glutamicum*, since the deletion of NCgl2816-*lldD* operon led to inability to grow on L-lactate as carbon and energy source, and complementation with a plasmid only carrying *lldD* in this deletion mutant recovered growth (Stansen et al., 2005).

Transcriptome analysis of L-lactate grown *C. glutamicum* showed high mRNA levels of NCgl2713 and NCgl2965, both encoding putative permeases (Stansen et al., 2005). L-lactate dehydrogenase is inducible, D-lactate dehydrogenase is constitutively expressed, but with a very low expression rate (Scheer et al., 1988). Therefore, the constitutive D-lactate dehydrogenase (*dld*) of the L-lysine producing *C. glutamicum* strain $lysC^{fbr}$ is a first obvious target for overexpression to increase D-lactate utilization of *C. glutamicum* (Scheer et al., 1988). The *ldhA* gene, encoding the NAD^+ dependent, soluble lactate dehydrogenase is used for reoxidation of NADH under conditions where oxidation by the respiratory chain is limiting and lactate is formed as a fermentation product (Inui et al., 2004b). Producer strains developed by classical methods often have uncharacterized mutations, leading to diminished stress tolerance, decreased substrate uptake or consumption whereas targeted engineering leads to robust production strains (Ohnishi et al., 2002). Elementary flux mode analysis and extreme pathway analysis (Papin et al., 2004), (Schilling et al., 2000), (Schuster et al., 1999) can be used to identify potential targets for directed metabolic engineering. First it permits the estimation of maximum possible yields (Krömer et al., 2006). Elementary flux mode analysis has also been used to correlate product fluxes with individual network fluxes to identify genes for overexpression in *C. glutamicum* and *A. niger* (Melzer et al., 2009). Flux balance analysis was used to study the impact of deletions and varying oxygen supply on *C. glutamicum* metabolic performance (Shinfuku et al., 2009). Several differing genome scale network models of *C. glutamicum* were recently published almost in parallel (Kjeldsen and Nielsen, 2009; Melzer et al., 2009; Shinfuku et al., 2009).

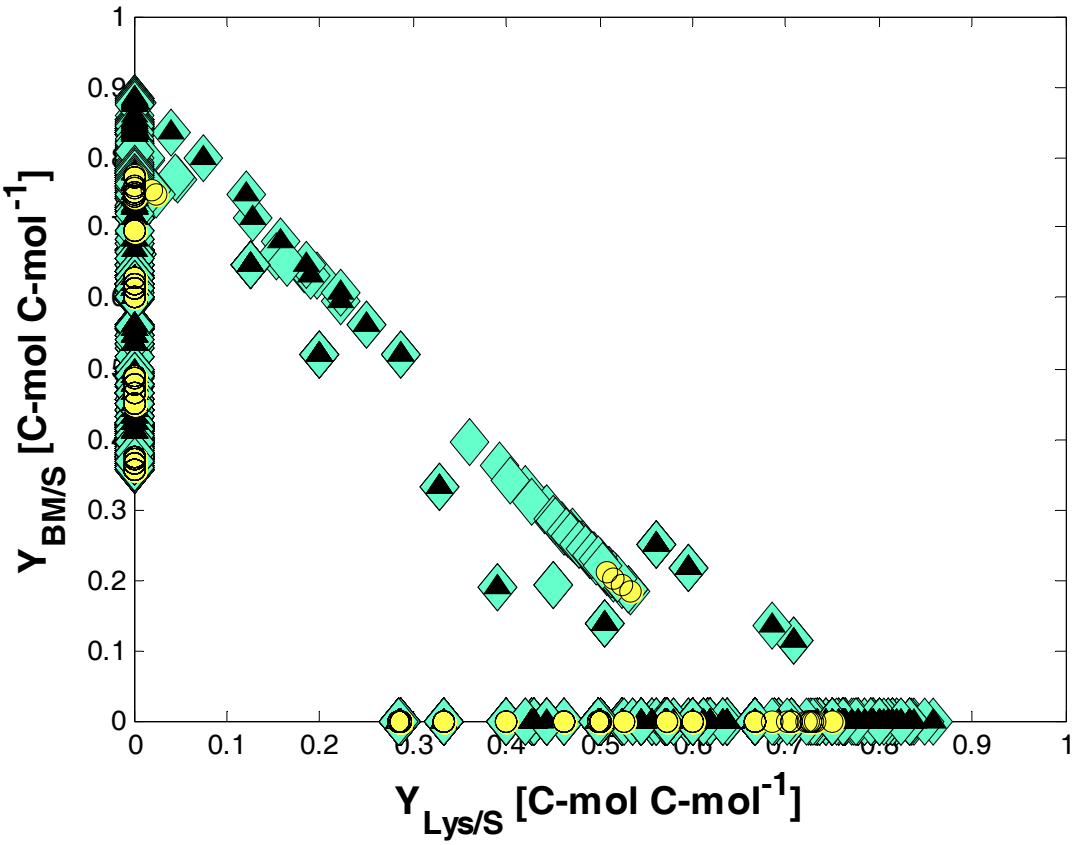


Figure 3.1: Carbon yields of biomass and L-lysine determined by elementary mode analysis using metabolic network of *C. glutamicum* (Supporting information, Figure S 3.1 and Table S 3.1). The network includes glucose uptake with PTS as well as via passive diffusional transport and glucokinase according to Shinfuku et al. (2009). It also comprises a reversible malic enzyme (open circles – elementary modes with growth only on lactate; full triangles – elementary modes with growth only on glucose; open diamonds – elementary modes with simultaneous consumption of lactate and glucose).

Since an effective NADPH supply has been identified as important prerequisite for superior L-lysine producers (Wittmann and Heinzle, 2002), the manner in which NADPH can be furnished during growth on substrates employing gluconeogenic pathways is of major interest (Cocaign-Bousquet and Lindley, 1995). To check to which extent L-lysine

yield during growth on mixed substrates is linked to an increased flux through the oxidative branch of the pentose phosphate pathway (PPP) (Wittmann and Heinzle, 2002), the deletion of glucose-6-phosphate isomerase gene is an interesting target (Marx et al., 2003).

In this work, elementary flux mode analysis was carried out for L-lysine production starting from published genome scale networks (Kjeldsen and Nielsen, 2009; Melzer et al., 2009; Shinfuku et al., 2009). It was used to investigate lactate, glucose and mixtures thereof as carbon sources for maximal yields of L-lysine. Flux modes representing high L-lysine production with a minimal enzyme requirement promise to deliver information which metabolic reactions and pathways are potential targets. This analysis predicted stimulation of L-lysine production by a combined overexpression of pyruvate carboxylase and malic enzyme. The present work describes overexpression of D-lactate dehydrogenase, pyruvate carboxylase and malic enzyme and also the deletion of glucose-6-phosphate isomerase and its impact on growth and L-lysine production on racemic lactate as well as on glucose-lactate mixtures.

Materials and Methods

Microorganisms: Bacterial strains and plasmids used in this study, their relevant characteristic and their sources of reference are listed in Table 3.1. All mutants were designed and constructed on the basis of the wild type *C. glutamicum* ATCC 13032 (American Type and Culture Collection) with deregulated L-lysine biosynthesis (allelic replacement of the *lysC* gene with a *lysC*T311I gene) (Kim et al., 2006). The vector used for introducing the modified genes, plasmid pClik is carrying a kanamycine resistance and the *sacB* gene as selective markers. The pClik plasmid has no origin of replication (ori) for *C. glutamicum*. Transformation of the organism with the plasmid and selection for the kanamycin resistance yielded transformants with genome integrated plasmid DNA. Integration of the plasmid DNA occurred via a single crossover homologous recombination. The second recombination was detected and selected via the *sacB* positive selection system (Jäger et al., 1992). Several sucrose resistant, kanamycin sensitive clones were tested for the presence of the mutation by PCR. Additionally, sequencing of the resulting PCR product was done. The primer sequences used for the verification of the promoter exchange upstream of the *dld*, *pyc* and *malE* genes as well as the corresponding fragment size for the wild type and the mutant alleles are listed in Table 3.2.

Table 3.1: Molecular biological tools and strains constructed starting from *C. glutamicum* ATCC 13032.

C. glutamicum $lysC^{fbr}$	Exchange T311I in the *lysC* gene	Kim et al. 2006
C. glutamicum $lysC^{fbr}dld_{Psod}$	lysC T311I + Exchange of the natural promoter of the *dld* gene by the promoter of the sod gene	This work
C.glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}$	lysC T311I + Exchange of the natural promoter of the *dld* and *pyc* genes by the promoter of the sod gene	This work
C. glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}$	lysC T311I + Exchange of the natural promoter of the *dld, pyc* and *malE* genes by the promoter of the sod gene	This work
C.glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}\Delta$ *pgi*	lysC T311I + Exchange of the natural promoter of the *dld, pyc* and *malE* genes by the promoter of the sod gene + deletion of the *pgi* gene	This work
E.coli DH5α	F^- *endA1, hsdR17 (vk^- mk^+) supE44, thi-I* λ^- *recAI gyrA96 rel A1*, Δ *(lac ZYA-argF)U169* F*80d lacZ* ΔM15	(Hanahan, 1983)
E.coli NM522	*supE thi-1* Δ*(lac-proAB)*Δ*(mcrB-hsdSM) 5(rK^- mK^+) [F' proAB lacI*q *Z*Δ*M15]*	Stratagene
Plasmids		
pClik int *sacB*	vector for integrative, allelic replacement by homologuous recombination, nonreplicative in *C. glutamicum*, Kan^R, *sacB*	BASF

Media: The first preculture was grown in complex medium containing 10 g L^{-1} glucose, 5 g L^{-1} yeast extract, 5 g L^{-1} beef extract, 5 g L^{-1} polypeptone, 20 g L^{-1} casaminoacids, 2.5 g L^{-1} NaCl and 2 g L^{-1} urea. For agar plates, 20 g L^{-1} agar were added. Second preculture and the main culture were grown in minimal medium (pH 7.2) containing (per litre): 10 g glucose, 10 g lactate, 16 g K_2HPO_4, 4 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 300 mg 3,4-dihydroxybenzoic acid, 10 mg $CaCl_2$, 250 mg $MgSO_4*7H_2O$, 10 mg $FeSO_4*7H_2O$, 10 mg $MnSO_4*H_2O$, 2 mg $ZnSO_4*7H_2O$, 200 µg $CuSO_4*5H_2O$, 20 µg $NiCl_2*6H_2O$, 20 µg $Na_2MoO_4*2H_2O$, 100 µg cyanocobalamin, 300 µg thiamine, 4 µg pyridoxal phosphate and 100 µg biotin.

Cultivation: Single colonies from an agar plate were used as inoculums for the first preculture, which was grown for 8 h at 30°C in a 100 ml baffled shake flask with 10 ml medium on a rotary shaker (Multitron II, Fa. Infors AG, 4103 Boltringen, Schweiz) at 230 rpm. Cells were harvested by centrifugation (3 min, 6500 x g, 4°C, Labofuge 400R, Heraeus, Hanau, Deutschland), washed twice with sterile 0.9% NaCl and used as inoculum for the second preculture, which was cultivated overnight under the same conditions. Main cultures were performed in duplicate using 250 ml baffled shake flasks with 25 ml medium. Cell concentration was determined by a photometer (Novaspec®II, Pharmacia Biotech, Little Chalfont, UK) at 660 nm. The correlation between cell dry weight (CDW) and the optical density at 660 nm was determined as described previously (Krömer et al., 2004).

Cell disruption: Cells were harvested by centrifugation (5 min, 6800 x g, 4°C). A small amount of glass beads (Ø 0.25 mm) and 1 ml MilliQ were added to the pellet. Disruption was performed using a bead mill (Retsch, MM301) at maximum speed (30 Hz) for 35 seconds. Cell debris was removed by centrifugation.

DNA preparation and transformation: Oligonucleotide synthesis was done by Sigma Aldrich. DNA sequencing by GATC, Konstanz, Germany. Chromosomal DNA from *C. glutamicum* was obtained using the Instant Bacteria DNA Kit (ANALYTIC JENA, Jena, Germany). Plasmids from *E. coli* were isolated using the GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, Chalfont St. Giles, Great Britain). *E. coli* was transformed by heat schock (Inoue et al., 1990). *C. glutamicum* was transformed by electroporation (van der Rest, 1999),(Tauch et al., 2002b). Cultivation of the *C. glutamicum* for electroporation was done in a 10 ml preculture, grown in BHI++ medium, inoculated from a fresh agar plate. The main culture (BHI++) was inoculated with the overnight preculture to an optical density of 0.2 at 660 nm. At an optical density around 0.6 400 mg isonicotinic acid hydrazide, 2.5 g glycine and 0.1 ml Tween 80 were dissolved in 20 ml H_2O and added to the cultivation medium filter sterilized. Cells were harvested at an optical density of 0.8 at 660 nm. Electroporation was performed with parameters set at 25 μF, 600 Ω and 2.5 kV. Immediately after electroporation, 1 ml BHIS medium was added to the cells and the suspension was transferred into a 2 ml Eppendorf tube for the following heatshock at 46°C

for 5 minutes. After the heatshock, the cells were incubated at 30°C for 90 minutes, to allow recovery and subsequently spread on $BHIS^{kan}$ agar plates.

DNA manipulations: All PCR reactions were done in a TGradient-Cycler (Whatman - Biometra®), with FidelyTaq (Fermentas, Hilden, Germany) or Jumpstart RedTaq Mix (SIGMA) using 10 mM dNTP Mix (Fermentas, Hilden, Germany) and the 1kb DNA O'GeneRuler™ (Fermentas, Hilden, Germany). Ligations were done with Fast-Link™ DNA Ligation Kit (Epicentre Biotechnologies, Madison, Wisconsin, USA). Restriction enzymes and their buffers were obtained from Fermentas (Hilden, Germany) and used as recommended by the manufacturers.

Overexpression of the *dld*, *pyc* and *malE* genes: Overexpression of the genes *dld*, *pyc* and *malE* in *C. glutamicum* was achieved by cloning the open reading frame (ORF) of the mentioned genes under the control of the promotor of the *sod* gene, encoding superoxide dismutase (NCgl2826). Wild type gene length and length after introduction of the sod promoter or deletion: *dld* 903/1095 bp, *pyc* 926/1118 bp, *malE* 917/1109 bp, *pgi* 2223/1820 bp.

Table 3.2: Primer sequences used for verification of the mutant strains and the PCR fragment size of the wild type and mutant alleles.

Genetic modification	Primer sequence	PCR fragment size
P_{sod}*dld*	Fw: 5'- GATCCTCGAGTCTGATTGCTGCGTCGATC-3'	WT: 903 bp
	Rw: 5'-GATCACGCGTCGAGTTGTTCGCGATG-3'	P_{sod}*dld*: 1095 bp
P_{sod}*pyc*	Fw: 5'-ATCGCTCGAGCTAATTTTTCTGAGTCTTAG-3'	WT: 926 bp
	Rw: 5'-CGATACGCGTGCCTTCACAAAGATGGGGTAAGTC-3'	P_{sod}*pyc*: . 1118 bp
P_{sod}*malE*	Fw: 5'-ATCGCTCGAGCTTACCAAGTGGG-3'	WT: 917 bp
	Rw: 5'-CGATACGCGTCGTGCATAACTGG-3'	P_{sod}*malE*: .1109 bp
Δ*pgi*	Fw: 5'-GATCACGCGTATCCCTTCTCCGGCATC-3'	WT: 2223 bp
	Rw: 5'-GATCTCTAGATCCAGCGACACGAATAATC-3'	Δ*pgi*: 1820 bp

D-lactate dehydrogenase and malic enzyme assay: For determination of enzyme activity, exponentially growing cells were harvested by centrifugation (5 min, 7000 rpm, 4°C) and washed three times with working buffer (50 mM ice cold Tris HCl, pH 7). After resuspension in 1 ml 50 mM Tris HCl, pH 7, cells were disrupted with a bead mill (Retsch, MM301) at maximum speed for 35 seconds. Cell debris was removed by centrifugation. Enzyme activity was measured in cell free supernatant. For the D-lactate dehydrogenase, reaction mixtures of 1.5 ml contained 1.4 ml 0.2 M Tris HCl, pH 7.3, 50 µl NAD^+(100mM) .The reaction was started by adding 50 µl 30 mM D-lactate. The reaction temperature was 25°C. For malic enzyme, the oxidative decarboxylation of malate contained 0.1 M KH_2PO_4 buffer, pH 7.8, 0.6 mM $NADP^+$, 5 mM $MgCl_2$ and 40 mM sodium L-malate. Enzyme activity was determined spectrophotometrically by measuring the absorbance change of NAD(P)H at 340 nm. Specific activity was calculated relative to the protein content of the cell extract. The protein content was quantified using the Bradford method (Bradford, 1976).The reagent solution was obtained from Biorad. Negative controls were performed without substrate or cell extract, respectively.

Analysis of substrates and products: Quantification of sugars and organic acids in 1:20 diluted supernatant was done by high pressure liquid chromatography (Kontron Instruments, Neufahrn, Germany) using a Aminex HPX-87H column (Bio-Rad, Hercules, Calif.) at 60°C with 7 mM H_2SO_4 mobile phase and a flow rate of 0.8 ml min^{-1}. Detection was via determination of refraction index (sugars) or UV absorption at 210 nm (organic acids). Quantification of amino acids was done as described by Krömer et al. (2005).

Metabolic reaction network: The metabolic network of *C. glutamicum* was set up for utilization of glucose and lactate based on the work done by Krömer et al.(2006) , using data from KEGG database (http:/ www.genome.jp/kegg/metabolism.html) as well as biochemical and physiological literature (Eggeling and Bott, 2005). It also incorporates collected knowledge from three recent publications of genome scale metabolic networks of *C. glutamicum* scale networks (Kjeldsen and Nielsen, 2009; Melzer et al., 2009; Shinfuku et al., 2009). It comprises glucose uptake via phosphotransferase system (PTS) and a putative permease combined with glucokinase (Shinfuku et al., 2009), lactate uptake via a permease system and lactate dehydrogenase, glycolysis pathway (EMP), pentose

phosphate pathway (PPP), tricarboxylic acid cycle (TCA), anaplerotic pathways including reversible malic enzyme (Kjeldsen and Nielsen, 2009), glyoxylate cycle, biomass production, L-lysine biosynthesis pathways and respiratory chain (supplementary Table S 3.1). For ATP production in the respiratory chain a P/O ratio of 2 (for NADH) and 1 (for FADH) was assumed (Klapa et al., 2003). The precursor demand for biomass formation was taken as described in the literature (Marx et al., 1996). Water, protons, phosphate and sulphur were not included in the balances. Putative glucose transport not using the PTS is described differently in the literature. While Kjeldsen and Nielsen (Kjeldsen and Nielsen, 2009) incorporated only energy driven transport of glucose either at the expense of ATP or protons, Shinfuku et al. (2009) used a simple permease that was also incorporated in the model here.

Computational methods: Elementary modes analysis was carried out using "efmtool", an open source program available at http:/www.csb.ethz.ch/tools/efmtool. The mathematical details of the algorithm are described in the literature (Terzer and Stelling, 2008). For each flux mode, the carbon yields of biomass ($Y_{x/s}$) and L-lysine ($Y_{Lys/s}$) were calculated as percentage of the substrate carbon.

Results and discussion

The two major carbon compounds found in grass silage, glucose and lactate (Krotscheck et al., 2004), were investigated as substrates, individually and as mixtures, for the production of L-lysine. To evaluate the potential of these two carbon sources, elementary mode analysis was carried out using the metabolic network depicted in Figure S 3.1 and Table S 3.1 of the supplementary material. The resulting carbon yields for biomass and L-lysine on pure glucose, pure lactate and also on mixtures of glucose and lactate are depicted in Figure 3.2.

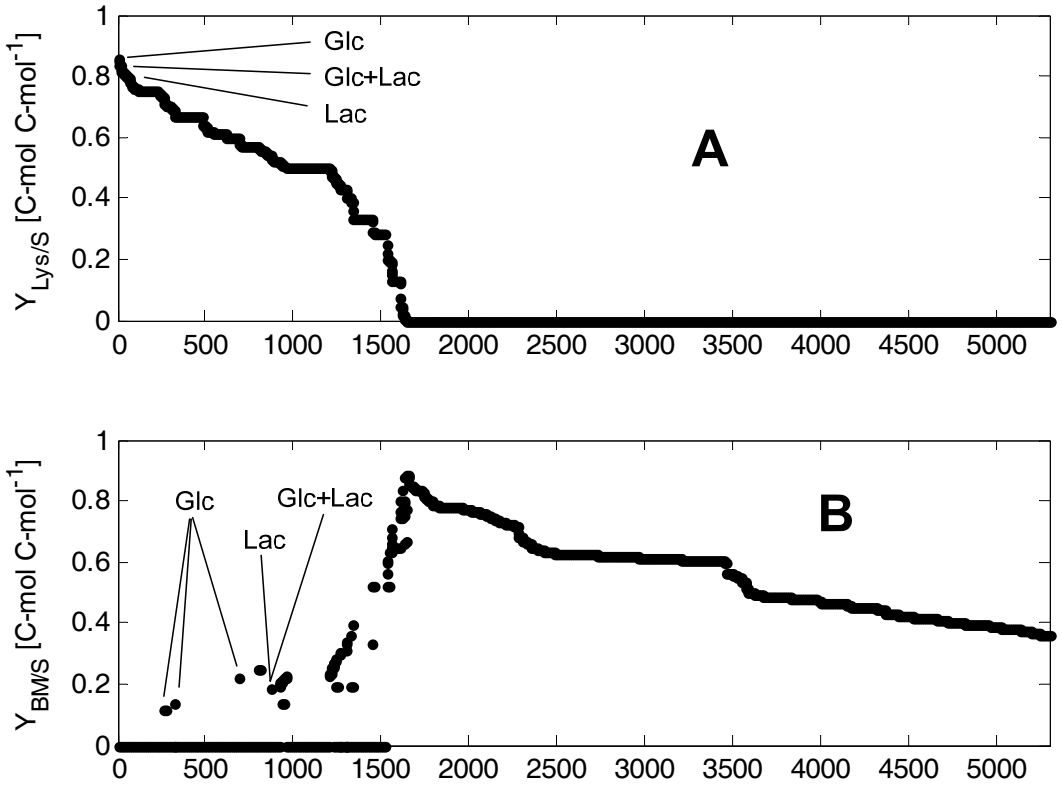


Figure 3.2: L-lysine and biomass yielding modes on mixtures of lactate and glucose sorted with carbon yield of L-lysine with first priority and biomass with second priority. A – carbon yield of L-lysine on glucose and lactate; B – carbon yield of biomass on glucose and lactate.

Many of these modes are so called extreme modes, producing either biomass or L-lysine exclusively, being displayed on the axes. In addition, numerous modes show simultaneous production of biomass and L-lysine. In Figure 3.2 all biomass and L-lysine yielding modes are plotted and sorted with respect to L-lysine yield as first priority and biomass yield as second priority. Maximal L-lysine carbon yield, $Y_{Lys/s}$, on lactate was 75%. Interestingly this mode used gluconeogenesis and NADPH production exclusively via glucose-6-phosphate dehydrogenase (R11) (Table S 3.2 of supplementary material). A best yielding mode excluding R11 is also listed there and resulted in 72.2%. In this mode NADPH was mostly produced by malic enzyme (R48) and to a lesser extent by isocitrate dehydrogenase (R32). Highest L-lysine carbon yield on glucose was 85.7% (Table S 3.3 of supplementary material), and this is larger than literature values (Kjeldsen and Nielsen, 2009; Melzer et al., 2009; Shinfuku et al., 2009). This mode was, however, not using PTS (R8) but only glucokinase (R60) that is usually not considered active in the wild type and produced equal amounts of NADPH by R11 and R48. It is a pure anaerobic mode where all electrons and ATP are completely balanced. Best mode after exclusion of R60 resulted still in 83.3%. Additional exclusion of malic enzyme (R48) gave a highest yield as described in the literature with 75%. For calculating the maximum yielding modes on glucose Kjeldsen and Nielsen (Kjeldsen and Nielsen, 2009) excluded malic enzyme activity achieving also a L-lysine yield of 75%. All the identified high yielding modes were using the dehydrogenase branch of L-lysine biosynthesis. The highest L-lysine carbon yield of an elementary flux mode with simultaneous consumption of glucose and lactate was 84.8% (Table S 3.4 of supplementary material). Regarding the modes with simultaneous biomass and L-lysine biosynthesis, the maximal, theoretical carbon yield for L-lysine with biomass production were 71% on glucose, 53% on lactate and 53% with simultaneous utilization of glucose and lactate with associated biomass carbon yields of 12%, 18% and 19%.

We used the identified elementary modes to get possible hints for useful targets for the engineering of *C. glutamicum*. The method presented by Melzer et al. (2009) plotting individual rates versus L-lysine production showed interesting correlations as depicted in the supplementary Figure S 3.2 but the correlation was rather poor with our network including glucokinase and malic enzyme activity.

Therefore we ranked the modes in different way using the objective function

$$OF = \sum_{i=1}^{n} \left(\frac{r_i}{r_{Lys} + r_{BM}} \right)^2 \quad (1)$$

Where r_i is any rate of the n reactions contained in the network listed in Table S 3.1 (supplementary material) and r_{Lys} and r_{BM} are the rates of production of L-lysine (R61) and biomass (R63) in that mode. By taking the square value, modes with multiple passages, e.g. in a cycle, are thus penalized. Modes with lowest objective function, *OF*, use most likely less enzymes and are thus expected to be more efficient than those with a high value of *OF*. This prediction is, however, not taking into account the unknown effect of different specific activities of all involved enzymes that may vary considerably. The resulting ranked values depicted in Figure S 3.3 show that low values of *OF* are generally correlating with high yields, both of L-lysine and biomass. In a next step we tried to identify possible targets for overexpression or deletion using the function shown below

$$Load_{i,j} = \sum_{j=1}^{n\,mode} \left(\frac{r_i}{r_{Lys} + r_{BM}} \right)_j Y_{Lys/S} \quad (2)$$

This function cumulates the rates of each reaction relative to the L-lysine and biomass production rates weighted with the L-lysine yield on both substrates of that mode. In Figure 3.3 it can be seen that for pure growth on lactate the most promising targets seem pyruvate carboxylase (R41) and malic enzyme (R48) since the cumulative function as defined in Equation 2 is initially increasing most significantly.

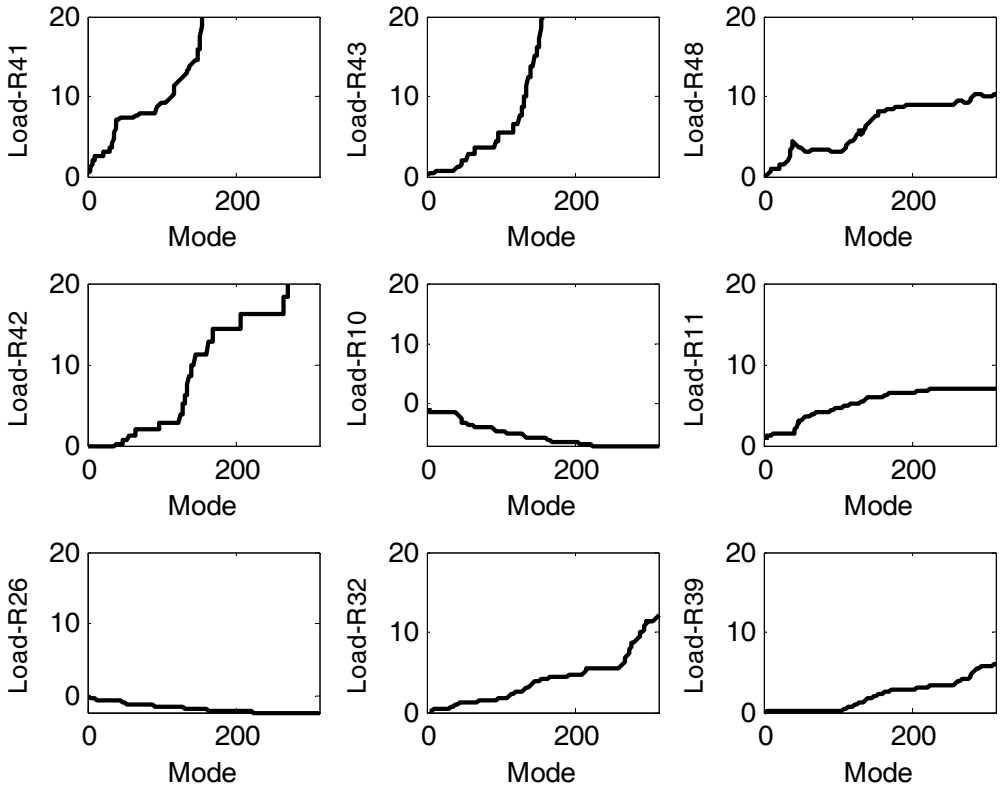


Figure 3.3: Elementary modes ranked with increasing objective function, *OF*, and selected cumulative enzyme loadings as defined in Eq. (2) for modes with pure growth on lactate. Reaction numbers are defined in Supporting information, Figure S 3.1 and Table S 3.1.

Interestingly, the second important group of genes is related to gluconeogenesis, e.g. phosphoenolpyruvate carboxykinase (R43) and glucose-6-phosphate isomerase (R10) and the pentose phosphate cycle, e.g. glucose-6-phosphate dehydrogenase (R11). Taking lactate (Figure 3.3) and all substrate combinations (supplementary Figure S 3.4 to S 3.6), pyruvate carboxylase is the most significant enzyme and malic enzyme, gluconeogenic and pentose phosphate pathway enzymes follow. These are therefore all possible targets for overexpression. For pure glucose consumption (supplementary Figure S 3.5) pyruvate carboxylase, malic enzyme and pentose phosphate pathway enzymes seem important while PEP carboxykinase seems less important and glucose-6-phosphate isomerase (R10) is nearly not required at all. In the analysis comprising all possibilities of substrate

consumption (supplementary Figure S 3.4) again pyruvate carboxylase and glucose-6-phosphate dehydrogenase seem very important but glucose-6-phosphate isomerase (R10) seems also relevant working in the reverse direction thus contributing to increased NADPH production. From this analysis we expected a reduction of L-lysine formation when glucose-6-phosphate isomerase was not operating. We checked the relevance of glucose-6-phosphate isomerase for the growth and L-lysine production on glucose-lactate mixtures by deleting it. Glyoxylate pathway is also a possible option for the production of L-lysine on lactate, but did not seem important using elementary flux mode analysis.

As a starting point, however, the most obvious target was constitutive overexpression of the D-lactate dehydrogenase (*dld*). *C. glutamicum* has two genes for lactate oxidation, encoding L–lactate dehydrogenase (*lldD*) and D–lactate dehydrogenase (*dld*). L-lactate dehydrogenase is inducible, D-lactate dehydrogenase is constitutively expressed at a very low rate (Cocaign-Bousquet and Lindley, 1995). *C. glutamicum* was engineered to improve growth on L and D- lactate mixtures, being able to metabolize the D-lactate fraction in a more efficient way.

Overexpression of the *dld, pyc* and *malE* genes.

The D-lactate dehydrogenase exhibited a low activity of 0.08 μmol (mg prot)$^{-1}$ min^{-1} in the parental strain *C. glutamicum lysC^{fbr}*, together with poor growth and incomplete lactate consumption (Table 3.3, supplementary Figure S 3.7). The D-lactate fraction was not metabolized. From the initial 10 g/L racemic lactate 4.3 g/L D- lactate remained until the end of cultivation as determined using an enzymatic test. In order to increase the *dld* expression, the native promoter of the gene was replaced by the strong promoter P$_{sod}$ from the gene *sod* of *C. glutamicum*, encoding the superoxide dismutase. The mutant strain *C. glutamicum lysC^{fbr}dld$_{Psod}$* showed increased *dld* activity of 0.42 ± 0.09 μmol (mg prot)$^{-1}$ min^{-1}. The same promoter was used for the overexpression of pyruvate carboxylase and malic enzyme. The specific activity of the malic enzyme was 0.21 ± 0.11 μmol (mg prot)$^{-1}$ min^{-1} in the parental strain and 0.96 ± 0.21 μmol (mg prot)$^{-1}$ min^{-1} in the mutant strain *C. glutamicum lysC^{fbr} dld$_{Psod}$ pyc$_{Psod}$ malE$_{Psod}$*. All genetic modifications were verified by

PCR, proving the integration of the desired constructs into the genome. Enzyme data proved that the use of a stronger promoter instead of the native one allowed an overexpression of the targeted genes.

Table 3.3: Growth characteristics of mutants of *C. glutamicum* ATTC created in this study. μ - specific growth rate; $Y_{X/S}$ – biomass yield on substrate given as g cell dry weight per C-mol of substrates; $Y_{Lys/S}$ – yield of L-lysine on substrates given in carbon moles per carbon mole.

Strain	μ [h^{-1}]		$Y_{X/S}$ [$^{g\ CDW}/_{C\text{-}mol\ S}$]		$Y_{Lys/S}$ [$^{C\text{-}mol\ Lys}/_{C\text{-}mol\ S}$]	
	Lactate	Gluc/Lac	Lactate	Gluc/Lac	Lactate	Gluc/Lac
lysC^{fbr}	0.22 ± 0.01	0.13 ± 0.01	0.18 ± 0.06	0.21 ± 0.07	0	0
lysC^{fbr} *dld*$_{Psod}$	0.34 ± 0.01	0.28 ± 0.02	0.21 ± 0.04	0.37 ± 0.02	0	0.05 ± 0.01
lysC^{fbr} *dld pyc* $_{Psod}$	0.38 ± 0.02	0.39 ± 0.01	0.29 ± 0.04	0.43 ± 0.07	0	0.08 ± 0.02
lysC^{fbr} *dld malE*$_{Psod}$	0.36 ± 0.01	0.28 ± 0.0	0.30 ± 0.02	0.38 ± 0.05	0.07 ± 0.01	0.11 ± 0.03
lysC^{fbr} *dld pyc malE*$_{Psod}$	0.29 ± 0.0	0.22 ± 0.02	0.23 ± 0.02	0.39 ± 0.07	0.09 ± 0.04	0.15 ± 0.01
lysC^{fbr} *dld pyc malE*$_{Psod}$ Δ*pgi*	-	0.26 ± 0.02	-	0.33 ± 0.06	-	0.13 ± 0.02

Effects on growth and L-lysine production on lactate.

The parental strain *C. glutamicum lysC*^{fbr} was cultivated on minimal medium with D,L-lactate as sole carbon and energy source (Figure S 3.7 of the supplementary material). The growth rate (μ = 0.22 h^{-1}) and the carbon yield of biomass of 18% were low whereby the strain was not able to utilize D-lactate effectively (Table 3.3). The specific lactate uptake rate calculated from data contained in Table 3.3 was 1.22 (C-mol S) (C-mol X)$^{-1}$ h^{-1}. As stated above, 4.3 g/l of D-lactate remained at the end of this culture from the original 10 g/l racemic lactate. The mutant *C. glutamicum lysC*^{fbr}*dld*$_{Psod}$ showed increased levels of D-lactate dehydrogenase activity and faster growth (μ = 0.34 h^{-1}) due to complete lactate consumption as well as an 30% increased specific uptake rate for D,L-lactate of qs_{lac} = 1.62 (C-mol S) (C-mol X)$^{-1}$ h^{-1} (calculated from Table 3.3). Additional overexpression of pyruvate carboxylase further increased the growth rate to μ = 0.38 h^{-1}. Enhancing the anaplerotic activity by the *pyc* overexpression obviously increased the supply of biomass precursors of the TCA cycle, glycolysis and pentose phosphate pathway as well as of NADPH resulting in a higher carbon yield of biomass of 29% (Table 3.3). Compared to the wild type strain the specific substrate uptake rate could be increased by 7% to qs_{lac} = 1.31 (C-mol S) (C-mol X)$^{-1}$ h^{-1}. Improved availability of precursor molecules and NADPH obviously led to the higher carbon yield of biomass and growth rate. Overexpression of malic enzyme in the *C. glutamicum lysC*^{fbr}*dld*$_{Psod}$*pyc*$_{Psod}$*malE*$_{Psod}$ strain led to a decreased growth rate of μ = 0.29 h^{-1} and biomass yield of 23%. The growth on lactate was exponential (Figure 3.4A) and well balanced with biomass and L-lysine production inversely proportional to lactate consumption (Figure 3.4B and 3.4C). This also goes along with a substrate uptake rate of qs_{lac} = 1.26 (C-mol S) (C-mol X)$^{-1}$ h^{-1}, 3% larger than that of the wild type strain, and a substantial shift in the distribution of the substrate carbon from biomass towards L-lysine production with a carbon yield of 13% as described in more detail later.

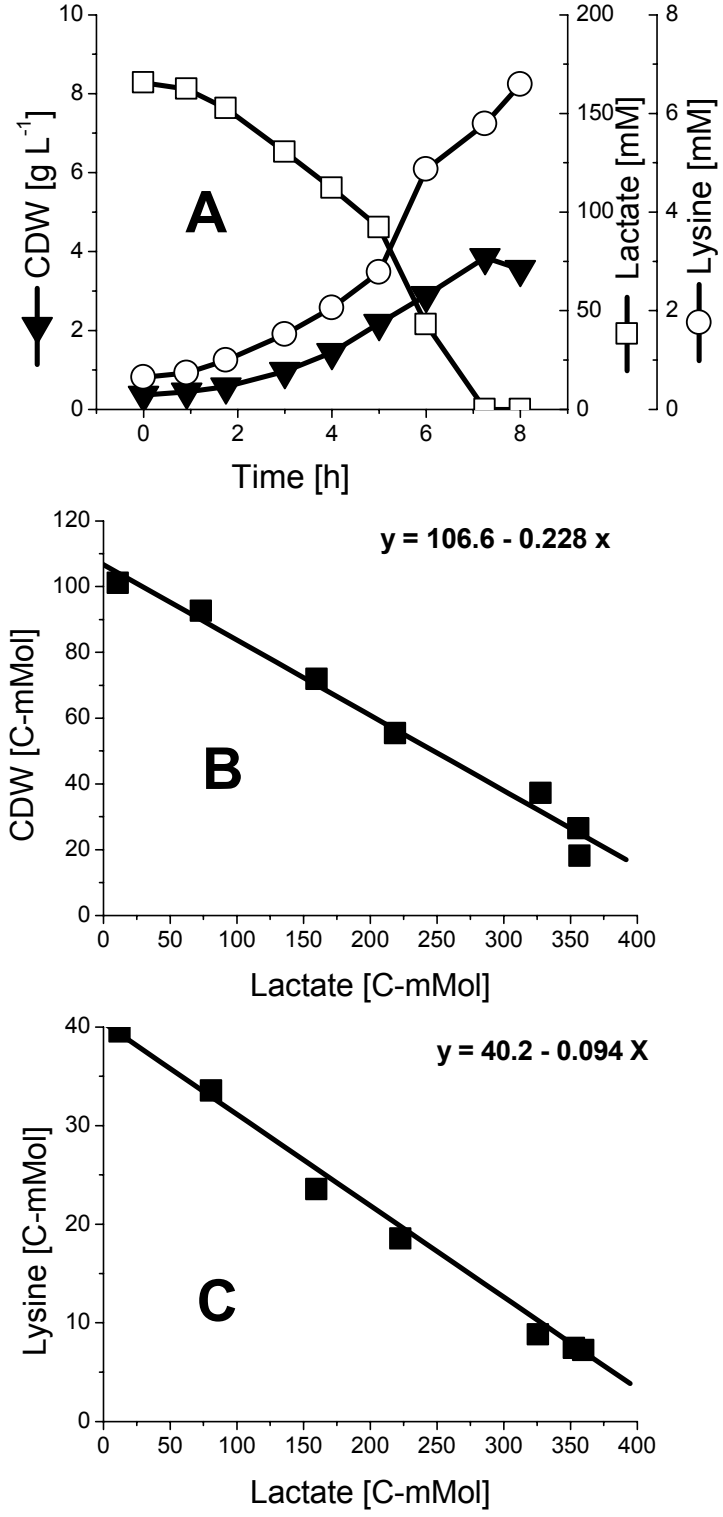


Figure 3.4: Growth and L-lysine production *C. glutamicum* ATCC 13032 *lysC*^{fbr}*dld*$_{Psod}$*pyc*$_{Psod}$*malE*$_{Psod}$ on D,L-lactate as sole carbon source. (A) Concentration profiles; (B, C) cell dry weight and L-lysine and lactate carbon concentrations.

Effects on growth and L-lysine production on lactate-glucose mixtures.

When grown on minimal medium with a glucose-lactate mixture as carbon source, the parental strain *C. glutamicum lysC^{fbr}* had a growth rate of $\mu = 0.13\ h^{-1}$ (Table 3.3). During growth on a glucose-lactate mixture lactate was metabolized at a specific rate equal to the case of pure lactate growth. Glucose was metabolized simultaneously but at a decreased consumption rate compared to pure glucose consumption (data not shown). After complete consumption of lactate, the glucose consumption rate increased back to that observed during growth on pure glucose. Similar observations concerning other organic acids are described in the literature (Georgi, 2006). The wild type strain had a poor D-lactate uptake that was even decreased in the presence of glucose yielding a value of $qs_{lac/glu} = 0.62$ (C-mol S) (C-mol X)$^{-1}$ h^{-1}. This is compatible with the slow growth of the *C. glutamicum lysC^{fbr}* on the glucose-lactate mixture. The *C. glutamicum lysC^{fbr}dld$_{Psod}$* strain showed a more than 100% increase of growth rate compared to the wild type with $\mu = 0.28\ h^{-1}$ (Table 3.3) and consumed lactate significantly faster with $qs_{lac/glu} = 0.76$ (C-mol S) (C-mol X)$^{-1}$ h^{-1}. Overexpression of the *pyc* gene further increased the growth rate to $\mu = 0.39\ h^{-1}$ with an even higher specific substrate uptake rate of $qs_{lac/glu} = 0.91$ (C-mol S) (C-mol X)$^{-1}$ h^{-1}. This strain also had the highest carbon yield on the lactate-glucose mixture of 43% (Table 3.3). *C. glutamicum lysC^{fbr}dld$_{Psod}$pyc$_{Psod}$malE$_{Psod}$* strain showed slower growth with $\mu = 0.22\ h^{-1}$ and lower substrate uptake $qs_{lac/glu} = 0.56$ (C-mol S) (C-mol X)$^{-1}$ h^{-1}. Carbon yield of biomass on the mixed substrates was 39% (Figure 3.5 and Table 3.3).

Lactate and glucose consumption were proportional to each other until complete consumption of lactate (Figure S 3.8 in supplementary material), interestingly with a nearly double carbon consumption rate of lactate (dLac/dGlc=1.96 C-mol C-mol^{-1}). Concurrent with the complete consumption of lactate, glucose uptake rate increased about threefold as can be seen from the slope in the plot of glucose versus biomass concentration in Figure S 3.8, where the slope equals the ratio of glucose and biomass rates. In the presence of lactate $Y_{X/Glc}$=1.15 (C-mol X) (C-mol Glc)$^{-1}$ and after consumption of lactate $Y_{X/Glc}$=0.42 (C-mol X) (C-mol Glc)$^{-1}$. Since the specific growth rate remained constant, the specific rate of glucose uptake increased by about 170%.

Effects on L-lysine overproduction.

Surprisingly, the parental strain *C. glutamicum lysC*^{fbr} did not produce detectable amounts of L-lysine neither on lactate nor lactate-glucose mixtures (Table 3.3). This is in contrast to the growth of this strain on pure glucose where a L-lysine carbon yield of 8.7% was obtained . During growth on lactate, the available NADPH and carbon were obviously primarily used for biomass formation though the aspartokinase is feedback resistant for L-lysine in this strain. A possible explanation of the absence of L-lysine secretion is the strong competition for oxaloacetate and NADPH by growth and L-lysine overproduction. This interpretation is supported by results obtained after overexpression of *dld* showing much faster lactate uptake but no beneficial effect on L-lysine production with lactate as sole carbon and energy source. Even overexpression of *pyc* that should increase the supply of oxaloacetate did not result in secretion of L-lysine during cultivation on lactate probably caused by a still limited supply of NADPH. On a lactate-glucose mixture the *C. glutamicum lysC*^{fbr}*dld*$_{Psod}$*pyc*$_{Psod}$ mutant with the additionally overexpressed pyruvate carboxylase had a L-lysine carbon yield of 8% and the highest biomass carbon yield of 43% (Table 3.3). The overexpression of *pyc* apparently leads to higher oxaloacetate formation, a key precursor for L-lysine production, thus increasing L-lysine carbon yield from 5% to 8%. The specific L-lysine production rate was nearly doubled from 0.038 to 0.073 (C-mol Lys) (C-mol S)$^{-1}$. Overexpression of malic enzyme in the *C. glutamicum lysC*^{fbr}*dld*$_{Psod}$*pyc*$_{Psod}$*malE*$_{Psod}$ increased the L-lysine yield on lactate from 0% to 9% and on lactate-glucose mixture from 8% to 15% corresponding to an 86% increase. Similarly, overexpressing malic enzyme in the *C. glutamicum lysC*^{fbr}*dld*$_{Psod}$ background increased L-lysine yield from 0% to 8.4% on lactate and from 5% to 11% on the lactate-glucose mixture, a 120% increase. This is most likely due to better NADPH supply, a key prerequisite for L-lysine biosynthesis. The specific production rates increased from 0.073 to 0.085 (C-mol Lys) (C-mol S)$^{-1}$, corresponding to 16% increase, and from 0.038 to 0.081 (C-mol Lys) (C-mol S)$^{-1}$ corresponding to a 113% increase (Table 3.3). Kinetics of this strain with highest L-lysine yield is shown in Figures 3.4 and 3.5. The growth on lactate was exponential and well balanced with biomass and L-lysine production inversely proportional to lactate consumption (Figure 3.4B and 3.4C). Interestingly biomass and L-lysine production remained constant even after complete exhaustion of lactate (Figure S 3.8 of supplementary material).

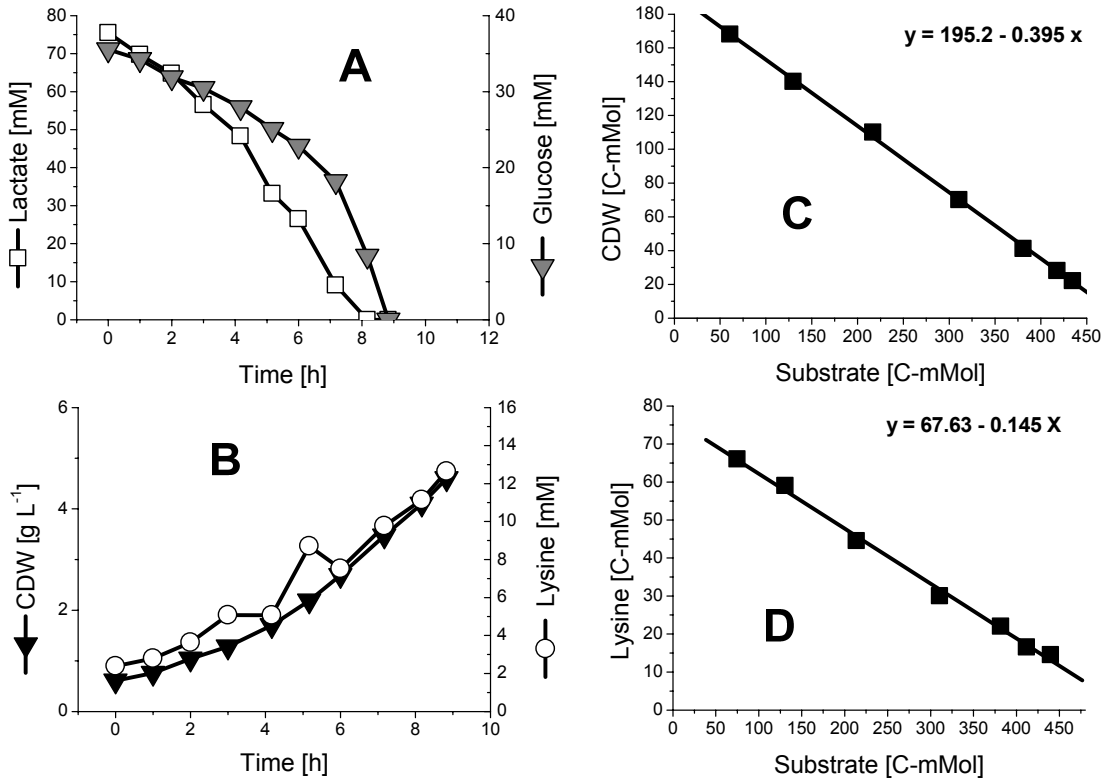


Figure 3.5: Growth and L-lysine production *C. glutamicum* ATCC 13032 *lysC*^{fbr}*dld*$_{Psod}$*pyc*$_{Psod}$*malE*$_{Psod}$ on a mixture of D,L-lactate and glucose. (A) Glucose and lactate concentrations; (B) cell dry weight (CDW) and L-lysine; (C, D) CDW and L-lysine and total substrate carbon concentrations.

Deletion of the phosphoglucose isomerase (*pgi*).

The elementary modes analysis predicted a substantial promotion of L-lysine yields on lactate by activation of gluconeogenesis and pentose phosphate but less with lactate-glucose mixtures (supplementary Figures S 3.4 to S 3.6). However, it was of great interest how significant the contribution of these pathways to L-lysine production was in a real strain producing L-lysine on glucose-lactate mixtures. To study the importance of gluconeogenesis and particularly the supply of NADPH by the oxidative branch of the pentose phosphate pathway, glucose-6-phosphate isomerase was deleted in the *C. glutamicum lysC*^{fbr}*dld*$_{Psod}$*pyc*$_{Psod}$*malE*$_{Psod}$ background. This deletion is channeling the whole glucose carbon flux through the PPP, with no split to glycolysis resulting in a production of two moles of NADPH per mole of glucose consumed. Deletion of *pgi* would,

however, not allow further carbon entering the oxidative part of the pentose phosphate pathway. Since improvement of the L-lysine yield is often linked to an increased flux through the PPP associated with high yield of NADPH (Wittmann and Heinzle, 2002) the glucose-6-phosphate isomerase seemed to be an interesting target to study the contribution of different pathways to the observed increased L-lysine production (Marx et al., 2003). The experimental results listed in Table 3.3 confirmed that glucose-6-phosphate isomerase is important for L-lysine overproduction on glucose-lactate mixtures and that its deletion is not beneficial for further improving the L-lysine yield. While the deletion of the *pgi* in the *C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}$ strain increased the specific growth rate to 0.26 h^{-1}, the L-lysine yield was reduced from 15% to 13%. Interestingly, however, the specific production rate of L-lysine increased to an observed maximum of all conditions studied here, i.e. 0.102 (C-mol Lys) (C-mol X)$^{-1}$.

Concluding remarks

In the present work, we have investigated the growth and L-lysine production of mutants of *C. glutamicum* ATCC 13032 *lysC*^{fbr}, serving as wild type strain in this study, on racemic mixtures of lactate as well as on mixtures of lactate and glucose. The growth on racemic lactate was significantly improved by the overexpression of *dld* both with respect to growth rate (55% increase) as well as biomass yield (17% increase). The growth rate increase is more than could be expected from a simple duplication of lactate supply to the metabolic network via pyruvate. Additional overexpression of *pyc* led to a further 12% increase of growth rate and a substantial 38% increase in biomass yield. Alternatively also the overexpression of malic enzyme in the *C. glutamicum lysC*^{fbr} *dld*$_{Psod}$ background led to a 6% increase in growth rate and 43% in biomass yield. The results in Table 3.3 show that for L-lysine production on lactate overexpression of malic enzyme is essential. The mutant *C. glutamicum lysC*^{fbr}*dld*$_{Psod}$ showed increased levels of D- lactate dehydrogenase activity of 0.42 µmol (mg prot)$^{-1}$ min^{-1}. Elementary mode analysis combined with the objective function of equation 1 and cumulative enzyme load calculated using equation 2 suggested pyruvate carboxylase as the most significant enzyme followed by malic enzyme but also gluconeogenic and pentose phosphate pathway enzymes. The overexpression of malic enzyme turned out to be essential for L-lysine production on lactate whereas the overexpression of *dld* alone did already result in 5% carbon yield in L-lysine. The parental strain gave a L-lysine yield of 8.7% on glucose whereas on the lactate-glucose mixture L-lysine was not produced at all. The best L-lysine yields were obtained in the *C. glutamicum lysC*^{fbr}*dld*$_{Psod}$*pyc*$_{Psod}$*malE*$_{Psod}$ strain, 9% carbon yield on lactate and 15% on a mixture of lactate and glucose. The predicted decrease of L-lysine yields caused by the *pgi* deletion could be experimentally confirmed. It seems that cyclic operation of the pentose phosphate pathway is significantly contributing to L-lysine production via an increased supply of NADPH as was previously observed for glucose, fructose and sucrose (Georgi et al., 2005; Kiefer et al., 2004; Wittmann et al., 2004).

All together we conclude that elementary flux mode analysis combined with useful objective functions permits a very useful prediction of potential targets for increasing L-lysine production by overexpression of selected genes. This may also be applied successfully in other cases, possibly combined with other useful methods as suggested by Melzer et al. (Melzer et al., 2009) but also the recently published method of Park et al. that

incorporated additional constraints of genomic context also of related organisms into target identification (Park et al., 2010). Our computational and experimental results also point to further potentially useful and scientifically interesting modifications, especially for enhancing gluconeogenesis and leading to a stronger flux through the PPP, e.g. overexpression of fructose 1,6-bisphosphatase (Georgi et al., 2005; Kiefer et al., 2004; Wittmann et al., 2004). Other interesting targets for further improvement of the constructed strains taken from the literature are the down – regulation of the TCA cycle by modifying start codons of TCA cycle genes to reduce their expression (Becker et al., 2009) or introducing a mutation in the malate quinone oxidoreductase (Ikeda et al., 2006). These modifications would lead to a decreased TCA activity thus avoiding unnecessary carbon loss. Application of genetically modified strains on real silage juice would require additional modifications to use other substrates contained in the juice, e.g. xylose.

Acknowledgements. We thank the Fachagentur für Nachwachsende Rohstoffe (FNR) that supported this work. We thank Prof. Roland Ulber of the University of Kaiserslautern for successful coordination of the joint project. We are very grateful for the most valuable support by Michel Fritz concerning instrumental analysis.

Conflict of interest statement. The authors have declared no conflict of interest.

Chapter 4

Production of L-lysine on different silage juices using genetically engineered *C. glutamicum**

*Published as: **Andreas Neuner**, Ines Wagner, Tim Sieker, Roland Ulber, Konstantin Schneider, Susanne Peifer and Elmar Heinzle (2012): Production of L-lysine on different silage juices using genetically engineered *Corynebacterium glutamicum*, *Journa of Biotechnology*, In Press.

Abstract

Corynebacterium glutamicum, the best established industrial producer organism for L-lysine was genetically modified to allow the production of L-lysine on grass and corn silages. The resulting strain *C. glutamicum* $lysC^{fbr}$ dld_{Psod} pyc_{Psod} $malE_{Psod}$ fbp_{Psod} $gapX_{Psod}$ was based on earlier work (Neuner and Heinzle, 2011). That mutant carries a point mutation in the aspartokinase (*lysC*) regulatory subunit gene as well as overexpression of D-lactate dehydrogenase (*dld*), pyruvate carboxylase (*pyc*) and malic enzyme (*malE*) using the strong Psod promoter. Here, we additionally overexpressed fructose 1,6-bisphosphatase (*fbp*) and glyceraldehyde 3-phosphate dehydrogenase (*gapX*) using the same promoter. The resulting strain grew readily on grass and corn silages with a specific growth rate of 0.35 h^{-1} and L-lysine carbon yields of approximately 90 C-mmol $(C\text{-mol})^{-1}$. L-lysine yields were hardly affected by oxygen limitation whereas linear growth was observed under oxygen limiting conditions. Overall, this strain seems very robust with respect to the composition of silage utilizing all quantified low molecular weight substrates, e.g. lactate, glucose, fructose, maltose, quinate, fumarate, glutamate, leucine, isoleucine and alanine.

Introduction

European green biorefinery concepts are usually based on silage guaranteeing a constant supply over the year (Hanegraaf et al., 1998; McDonald et al., 1991). Ensiling is the process of preserving the wet plant material applying anaerobic conditions, either in a storage silo or wrapped in plastic. After harvest, the green plant material is chopped and left to wither up to a dry mass content of approximately 30% followed by the removal of oxygen by compression assuring anaerobic conditions. Lactic acid bacteria convert a large fraction of water soluble carbohydrates into lactic acid. The low pH (3-4) and the anaerobic conditions prevent coliform bacteria and clostridia from spoiling the crop (McDonald et al., 1991). On a large scale, silage is either used for the production of biogas or as animal feed (Kromus et al., 2004). In the case of biogas production it seems interesting to convert part of the easily accessible compounds into more valuable products, e.g. amino acids, and use the remainder for biogas production. We aimed at the production of L-lysine using *Corynebacterium glutamicum* (Eggeling and Bott, 2005). Since *C. glutamicum* is a GRAS organism, fermentation broth containing L-lysine and *C. glutamicum* could be used as feed supplement for monogastric animals in close-by chicken and pig farming (Leuchtenberger et al., 2005). A second alternative might be a direct combination of L-lysine fermentation of silage to enrich silage with L-lysine. Only small increases in L-lysine content improves the biological value measured by the protein efficiency ratio significantly (Belitz, 2001). This makes L-lysine the most used amino acid in animal feed supplementation (Belitz, 2001) improving the nitrogen uptake, enhancing growth (Leclercq, 1998) and decreasing the release of nitrogen into the environment.

Dominating compounds in sugar beet and corn silages are sucrose and starch, respectively, whereas grass silage does not have a major component. Lactic acid itself is a valuable intermediate for the manufacture of biodegradable polymers (Södergard, 2002) but recovery costs from silage are very high (Datta et al., 1995). Therefore, we try to use it as an carbon source for L-lysine production (Neuner and Heinzle, 2011).

C. glutamicum, a gram positive, non-pathogenic soil bacterium grows aerobically on various carbohydrates and organic acids as carbon sources (Liebl et al., 1991) but only poorly on racemic lactate (Neuner and Heinzle, 2011). Applying metabolic network analysis using elementary modes (Kjeldsen and Nielsen, 2009; Krömer et al., 2006; Melzer et al., 2009; Neuner and Heinzle, 2011; Schuster et al., 1999) to guide genetic engineering, *C. glutamicum* was engineered to grow on racemic lactate and on carbohydrates and mixtures thereof and at the same time producing L-lysine (Neuner and Heinzle, 2011).

In this work, the L-lysine producing strain *C. glutamicum* $lysC^{fbr}$ dld_{Psod} $pyc_{Psod}$$malE_{Psod}$ (Neuner and Heinzle, 2011) was further genetically modified and tested for efficient L-lysine production on different silage juices. We focused on the three main carbon sources, glucose, fructose and lactate. However, other sugars and organic acids existing in silage juices (Wiseman and Irvin, 1957) were also analyzed. Besides earlier identified and tested modifications (Neuner and Heinzle, 2011) we studied two additional targets for overexpression that were predicted by the same model and method but using lactate, glucose and fructose as substrates. The gluconeogenic enzyme fructose 1,6-bisphosphatase (*fbp*) was already earlier found being beneficial for L-lysine production on carbohydrates (Becker et al., 2005; Becker et al., 2011; Rittmann et al., 2003) and therefore was constitutively overexpressed. Furthermore, glyceraldehyde 3-phosphate dehydrogenase that was also predicted as beneficial for L-lysine production was overexpressed. We studied growth and L-lysine production of the resulting strain on different silage juices and under different cultivation conditions and found substantial L-lysine production even under oxygen limitation as was already observed previously (Ensari and Lim, 2003).

Material and methods

Pretreatment of silage juice

The silages we used were provided by the Lehr- und Versuchsanstalt für Viehhaltung, Hofgut Neumühle (Münchweiler an der Alsenz, Germany). The silage juices were obtained by using a HP2H tincture press (Fischer Maschinenfabrik GmbH, Neuss, Germany). We applied filtration with stericups (Merck Millipore, Darmstadt, Germany) and pasteurization, using different temperatures for different periods of time. When using filtration with 0.22 µm pore size, the silage juices were centrifuged previously at maximum speed (16,000 × *g*) for 10 minutes to remove the majority of the particles from the suspension. The pH was adjusted to a value of 7 using 30% ammonia solution or 2 N NaOH. Heat pre-treatment tests were only made on grass silage. After the heat pre-treatment, silage juice was plated out on LB and CM agar plates and incubated at 30°C and 37°C for one week. Colony formation was observed. Subsequently, the treated juice was used for growth experiments with *C. glutamicum* mutants.

Strains, plasmids and recombinant DNA methods

All mutants were designed on basis of the L-lysine producing strain *C. glutamicum* ATCC 13032 *lysC*^{fbr} (BASF AG, Ludwigshafen, Germany) with deregulated L-lysine biosynthesis (allelic replacement of the *lysC* gene with a *lysC*T311I gene) (Kim et al., 2006). Overexpression of the genes *fbp* and *gapX* in *C. glutamicum* was achieved by cloning the open reading frame (ORF) of the mentioned genes under the control of the strong constitutive promoter of the *sod* gene, encoding superoxide dismutase (NCgl2826).

E. coli DH5α was used for the amplification of the genetic constructs. Application of the methylation pattern of *C. glutamicum* to the genetic constructs was performed using *E. coli* NM522, containing the pTc plasmid as an expression vector for the DNA –

methyltransferase of *C. glutamicum*. The integrative plasmid pClik int *sacB*, carrying a kanamycin resistance and the *sacB* gene as selective markers was used for introducing the genetic modifications. Transformation of the organism with the plasmid and selection for kanamycin resistance yielded transformants with genome integrated plasmid DNA. Integration of the plasmid DNA occurred via a single crossover homologous recombination. The second recombination was detected and selected via the *sacB* positive selection system (Jäger et al., 1992). Sucrose resistant, kanamycin sensitive clones were tested for the presence of the mutation by PCR. In addition, sequencing of the resulting PCR product was performed (GATC, Konstanz, Germany). Detailed information about all bacterial strains and plasmids used in this study, their relevant characteristics and their sources are listed in Table 4.1. Used primers are listed in Table 4.2.

Table 4.1: Molecular and biological tools and strains constructed starting from C. glutamicum ATCC 13032.

Strain	Modifications	Reference
C. glutamicum $lysC^{fbr}$	Exchange T311I in the *lysC* gene	BASF
C. glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}$	lysC T311I + Exchange of the natural promoter of the *dld, pyc* and *malE* genes by the promoter of the *sod* gene	Neuner and Heinzle, 2011
C. glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}$	lysC T311I + Exchange of the natural promoter of the *dld, pyc, malE, fbp* genes by the promoter of the *sod* gene	This work
C. glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}gapX_{Psod}$ Referred to as *C. glutamicum* strain SL.	lysC T311I + Exchange of the natural promoter of the *dld, pyc, malE, fbp, gapX* genes by the promoter of the *sod* gene	This work
E.coli DH5α	F^- *endA1, hsdR17 (*vk^- mk^+*) supE44, thi-I* λ^- *recAI gyrA96 rel A1*, Δ *(lac ZYA-argF)U169* F*80d lacZ* ΔM15	(Hanahan, 1983)
E.coli NM522	*supE thi-1* Δ*(lac-proAB)*Δ*(mcrB-hsdSM) 5(*rK^- mK^+*) [F' proAB lacl*q *ZΔM15]*	Stratagene
Plasmid		
pClik int *sacB*	vector for integrative, allelic replacement by homologuous recombination, nonreplicative in *C. glutamicum*, Kan^R, *sacB*	BASF

Table 4.2: Primer sequences used for verification of the mutant strains and the PCR fragment size of the wild type and mutant alleles.

Genetic modification	Primer sequence	PCR fragment size	
P_{sod}*gapX*	Fw: 5'- ATCGACGCGTTCGCAGCCGGCGGCCTTTCAACCTCCG - 3'	WT:	1218 bp
	Rw: 5' - CGATCTCGAGCGCCAGCGGCCGGTGTTGTCTACCACGACG - 3'	P_{sod}*dld*:	1410 bp
P_{sod}*fbp*	Fw: 5'- AGTTGCATGATCAGTCATTGCGCGCGCTTCC -3'	WT:	1377 bp
	Rw: 5'- AGTCTGTCCACCAGCTGTCCAAGCTGCAGGAATAC - 3'	P_{sod}*pyc*:	1569 bp

For the construction and purification of plasmid DNA standard protocols were applied. Chromosomal DNA from *C. glutamicum* was obtained using the Instant Bacteria DNA Kit (Analytic Jena, Jena, Germany). Plasmids from *C. glutamicum* were isolated using the HiSpeed® Midi Kit (Quiagen, Hilden, Germany). Plasmids from *E. coli* were recovered using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Munich, Germany). Oligonucleotide synthesis was carried out by Sigma (Munich, Germany). All PCR reactions were done in a TGradient-Cycler (Whatman - Biometra®, Goettingen, Germany), with FidelyTaq (Fermentas, Mannheim, Germany) or Jumpstart RedTaq Mix (Sigma, Munich, Germany) using 10 mM dNTP Mix and the 1kb DNA O'GeneRuler™ (Fermentas, Mannheim, Germany). Ligations were performed with Fast-Link™ DNA Ligation Kit (Epicentre Biotechnologies, Madison, USA). Restriction enzymes and their buffers were obtained from Fermentas (Mannheim, Germany) and used as recommended by the manufacturers. *E. coli* was transformed by heat shock. *C. glutamicum* was transformed by electroporation (Tauch et al., 2002b; van der Rest, 1999). Strain verification was done by DNA sequencing, GATC, Konstanz, Germany.

Media

For the bioreactor cultivations, filtered silage juice was diluted with water (1:4; v:v) and then directly used as fermentation broth. For sterility tests we used LB (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, 20 g/L agar, pH 7) and CM (10 g/L glucose, 2.5 g/L NaCl, 2 g/L urea, 5 g/L yeast extract, 5 g/L peptone, 20 g/L casaminoacids, 20 g/L agar, pH 7) agar plates. Genetic manipulations of *C. glutamicum* have been performed using different complex media containing 10 g L^{-1} glucose, 5 g L^{-1} yeast extract, 5 g L^{-1} beef extract, 5 g

L^{-1} polypeptone, 20 g L^{-1} casaminoacids, 2.5 g L^{-1} NaCl and 2 g L^{-1} urea. The selection of positive mutants after the first recombination was done on CM^{Kan} agar plates,containing 20 mg/ml kanamycine. The selection after the second recombination on CM^{Sac} was made on agar plates containing 100 g L^{-1} sucrose. For agar plates, 20 g L^{-1} agar were added. After electroporation, cells were incubated in BHIS medium containing 37 g L^{-1} BHI and 250 ml 2M sorbitol solution for recovery. All other chemicals and reagents of analytical grade were purchased from Sigma-Aldrich, Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland) or Roth (Karlsruhe, Germany).

Cell disruption

Cells were harvested by centrifugation (5 min, 6,500 x *g*, 4°C). 100 mg of glass beads (Ø 0.25 mm) and 1 mL deionized purified water were added to the pellet. Disruption was performed using a bead mill (Retsch, MM301, Haan, Germany) at 30 Hz for 35 seconds. Cell debris was removed by centrifugation.

Fructose 1,6-bisphosphatase activity

The *in vitro* FBPase activity in cell extracts of *C. glutamicum* was measured in a coupled spectrophotometric assay containing 100 mM Tris/HCl, 5 mM $MnCl_2$, 0.5 mM $NADP^+$, 2 U/mL of phosphoglucoisomerase, 1 U/mL of glucose 6-phosphate dehydrogenase, pH 7.7, 30°C, 50 µl cell extract for the reaction mix and 100 mM fructose 1,6-bisphosphate, 100 mM Tris/HCl, pH 7.7 for the substrate solution. Temperature optimum was determined to be 30°C. The assay was started by adding 50 µl substrate solution to 950 µl reaction mix. Fructose 6-phosphate formed by the reaction of FBPase was converted to glucose 6-phosphate and subsequently to 6-phosphogluconate by phosphoglucoisomerase and glucose 6-phosphate dehydrogenase. The formation of NADPH ($\varepsilon_{340\ nm} = 6.22\ mM^{-1}\ cm^{-1}$) was monitored at 340 nm. Negative controls were carried out without fructose 1,6-bisphosphate or without cell extract, respectively. All measurements were performed in triplicate.

Glyceraldehyde 3-phosphate dehydrogenase activity

The *in vitro* activity of glyceraldehyde 3-phosphate dehydrogenase was determined according to a published method (Crow and Wittenberger, 1979). The reaction mixture contained 1 mM NAD^+, 5 mM sodium arsenate, 5 mM cysteine/HCl, 125 mM triethanolamine and cell extract, pH 7.5. The substrate mixture contained 2 mM D,L-glyceraldehyde 3-phosphate. Glyceraldehyde 3-phosphate dehydrogenase was assayed after 1 h incubation in triethanolamine/HCl buffer, containing cysteine/HCl to reactivate the enzyme by reducing sulfhydryl groups. The assay was initiated by the addition of glyceraldehyde 3-phosphate. The assay is based on the coupling of enzyme activity to the consumption or production of NADH, monitored at 340 nm in a spectrophotometer ($\varepsilon_{340\ nm}$ = 6.22 mM^{-1} cm^{-1}). All measurements were performed in triplicate. Negative controls were carried out without substrate or without cell extract, respectively. All measurements were performed in triplicate. Protein concentration was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard.

Shake flask cultivation

Shake flask cultivations were performed using undiluted, filtrated grass and corn silage juice, respectively. The precultures and main cultures in shake flasks were performed using the same medium, undiluted silage juice. Single colonies from agar plates were used as inoculums for the first preculture, which was grown for 8 h at 30°C in a 100 mL baffled shake flask with 10 mL medium on a rotary shaker (Multitron II, Infors AG, Bottmingen, Switzerland) at 230 rpm. Cells were harvested by centrifugation (3 min, 6,000 rpm, 4°C, Labofuge 400R, Heraeus, Hanau, Germany) and used as inoculum for the main culture. Main cultures were performed in duplicate using 250 mL baffled shake flasks with 25 mL medium. Cell concentration was determined photometrically (Novaspec®II, Pharmacia Biotech, Little Chalfont, UK) at 660 nm.

Bioreactor cultivation

Pre-cultures and main cultures were performed in 1:4 (v:v) diluted silage juice, providing all necessary macro- and micronutrients. Cultivations were performed in a 2.3 L bioreactor

RK 01 – 23, (FairMenTech GmbH, Göttingen, Germany) with 1000 mL working volume at 30°C, pH 7.0, 800 rpm, unless stated otherwise, The PEEK stirrer shaft diameter was 10 mm and the outer diameter ring carrying the blades 16 mm. The ring carried six impeller blades of 10 mm height and 3 mm thickness. The total impeller diameters were 35 mm for the small (impeller 1) and 52 mm for the large impeller (impeller 2). A three stage stirrer was used. Dissolved oxygen was measured via a pO_2 probe (Broadley –James Corporation, Irvine, USA). Aeration rates were adjusted to meet the oxygen requirements of the strains using a mass flow controller (Brooks Instruments, Veenedaal, Netherlands). From measured gas flow rates and composition of aeration and exhausted gas (quadrupole mass spectrometer Omnistar, Balzers AG, Vaduz, Liechtenstein) oxygen uptake (OUR) and CO_2 production rates (CPR) as well as the respiratory quotient (RQ) were determined on line (Heinzle et al., 1990). During cultivation the pH was adjusted with 2 N HCl and 2 N NaOH.

Analysis of substrates and products

Cultivation samples were centrifuged (3 min, 13,000 x *g*, 4°C, Heraeus, Hanau, Germany) and the supernatant was used for the analysis of substrates and products. Quantification of sugars and organic acids in diluted supernatants was performed by high pressure liquid chromatography (Kontron Instruments, Neufahrn, Germany) using a Aminex HPX-87H column (Bio-Rad, Hercules, Calif.) at 60°C with 7 mM H_2SO_4 mobile phase and a flow rate of 1 mL min^{-1}. Detection was carried out using a refraction index detector (sugars) or a UV detector (210 nm, organic acids). Quantification of amino acids was performed as described by Krömer et al. (2005). Alternatively, enzyme kits (Boehringer Mannheim, R Biopharm AG, Darmstadt, Germany) were used for the quantification of D,L-lactate (D,L-lactate UV – test) and glucose / fructose (D-Glucose / D-fructose UV – test).

Results and discussion

Pretreatment of silage juice

Heat pretreatment experiments were carried out using grass silage. 1 kg of silage yielded approximately 550 mL silage juice, depending on the silage. pH was adjusted to a value of about 7 using 30% ammonia solution or NaOH. Autoclaving the silage juice (20 min, 121 °C) obviously produced inhibitory or even toxic compounds, presumably by the Maillard reaction. Since the process was designed for a potential future application in farming units and biorefineries, we examined pasteurization to minimize spoilage. The pasteurization parameters were tested by exposing the silage juice to different temperatures for different periods of time. By determination of the growth rate and comparison with growth rates obtained on filtrated juice, we elucidated the optimal time and temperature for the pasteurization of silage juice. The results are depicted in Table 4.3. As shown in Table 4.3, even short treatment of 5 min at temperatures of 100°C caused growth inhibition. Since silage juice contains amino acids and sugars, excess heat causes the Maillard reaction to occur. In an alkaline environment this reaction is accelerated as the amino groups are deprotonated and therefore more nucleophile. The pH of silage juice was adjusted to a value of 7, and we applied temperatures of over 80°C. This is a possible explanation for the growth inhibition after extended exposure of the silage juice to this temperature. For the determination of the specific growth rate we inoculated with a higher OD_{660} of 2 in cases of non-sterile silage juice (80°C for 5 and 10 minutes). Therefore, the measured growth rate can be attributed to the clearly predominant organism, *C. glutamicum*. The results of Table 4.3 show that pasteurization at 80°C for 15 min is sufficient to avoid spoilage by undesired microorganisms and at the same time to guarantee maximum growth. This kind of pretreatment could be easily implemented on a larger scale using a counter-current heat exchanger system.

Table 4.3: Heat treatment of the grass silage juice with resulting sterility and growth characteristics. Sterility was tested by plating treated silage on LA and CM agar plates and incubation at 30 °C and 37 °C for seven days. Sterility: sterile – no colonies on any plate; not sterile – colonies observed. Growth of *C. glutamicum* was tested in shake flask cultures.

Temp / Time	5 min	μ [h^{-1}]	10 min	μ [h^{-1}]	15 min	μ [h^{-1}]
80°C	not sterile		not sterile		sterile	
	growth	0.35±0.02	growth	0.34±0.03	growth	0.36±0.01
100°C	not sterile		sterile		sterile	
	growth	0.26±0.01	no growth	-	no growth	-
120°C	sterile		sterile		sterile	
	no growth	-	no growth	-	no growth	-

Validation of the *fbp* and *gapX* overexpression

To validate the overexpression of the *fbp* and *gapX* genes, the *in vitro* specific enzyme activities in the precedent strain, *C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}$ were compared to the specific activities in the *C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}$ and *C. glutamicum* $lysC^{fb}$ $dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}gapX_{Psod}$ mutants. Results are displayed in Table 4.4. The activity data show fourfold increased activity of the fructose 1,6-bisphosphatase and a nearly 2.5-fold increased activity of glyceraldehyde 3-phosphatase in the new strain, proving a successful overexpression of the *fbp* and *gapX* genes.

Table 4.4: Enzyme activities of fructose 1,6-bisphosphatase, fbp, and glyceraldehyde 3-phosphate dehydrogenase, gapX, in various strains.

Enzyme	Strain	Enzyme activity (mU/mg protein)
fbp	*C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}$	21.3 ± 0.4
	C. glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}$	86.1 ± 2.6
gapX	*C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}$	568 ± 16
	C. glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}gapX_{Psod}$	1318 ± 29

Influence of *fbp* and *gapX* overexpression on L-lysine production

C. glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}$ was able to produce L-lysine on grass silage juice, with a L-lysine carbon yield of 3.2 ± 0.2%.The additional overexpression of the *fbp* gene in the mutant *C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}$ resulted in an almost threefold increased L-lysine carbon yield of 9.0 ± 1.1%. This proves the importance of the *fbp* gene regarding L-lysine production, especially when gluconeogenic substrates are used. Former studies showed a L-lysine carbon yield increase of 30% during growth on fructose minimal medium after the overexpression of the fructose 1,6-bisphosphatase (Becker et al., 2005). Using silage with lactate as main carbon source and fructose in considerable amounts, this gluconeogenic enzyme plays an even more important role, increasing the L-lysine yield threefold. Additional overexpression of *gapX* slightly improved the L-lysine carbon yield to 9.4 ± 0.2% that was however not statistically significant.

Influence of the *fbp* and *gapX* overexpression on growth

Besides strongly affecting the L-lysine production, the overexpression of the *fbp* gene also influenced the specific growth rate and biomass formation. Compared to the *C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}$ strain (μ = 0.38 h^{-1}), the specific growth rate μ slightly decreased in the *C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}$ mutant (μ = 0.35 h^{-1}).

The *C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}$ mutant exhibited a 20% higher biomass carbon yield (44%) than the precedent strain (36%). A possible reason is an increased flux through the pentose phosphate pathway, generating more NADPH that boosted L-lysine production but also lead to an increased growth requiring NADPH as well. Former studies described a comparable increase of biomass formation on fructose of about 10% using a strain overexpressing *fbp* (Becker et al., 2005). Additional overexpression of *gapX* resulted in an almost identical growth rate (μ = 0.36 h^{-1}) but reduced biomass carbon yield of 38%.

Influence of the *fbp* and *gapX* overexpression on byproduct formation

Especially on grass silage juice, containing a significant amount of fructose, dihydroxyacetone (DHA) was a byproduct secreted by the *C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}$ mutant. Towards the end of the cultivation, the DHA reached a final level of 7.1 ± 0.1 mM. Overexpression of the fructose 1,6-bisphosphatase in the *C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}$ mutant decreased the DHA level by almost 50%. Additional overexpression of glyceraldehyde 3-phosphate dehydrogenase further decreased the DHA secretion to 1.9 ± 0.1mM. In corn silage, byproduct formation in form of DHA was insignificant. The results showing the effect of the *fbp* and *gapX* overexpression on DHA formation on grass silage juice are depicted in Table 4.5.

Table 4.5: Formation of dihydroxyacetone in various mutants.

Strain	Dihydroxyacetone (mM)
C. glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}$	7.1 ± 0.1
C. glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}$	3.2 ± 0.2
C. glutamicum $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}fbp_{Psod}gapX_{Psod}$	1.9 ± 0.1

Cultivation experiments

Bioreactor cultivation

In all cultivation experiments applying shake flasks and bioreactors profiles of major carbohydrates, lactate, L-lysine and biomass were determined. Additional carbon sources like the organic acids quinate and fumarate as well as the four predominant amino acids in silage juice, i.e. glutamine, leucine, isoleucine and alanine were determined at the beginning and at the end of each experiment since their amounts were significantly lower (Table 4.6).

Table 4.6: Initial concentration of minor substrates measured. In all cultivations all these substrates were completely consumed at the end of the respective experiment.

	Concentration [C-mmol L^{-1}]				
	Grass silage juice			Corn silage juice	
	F1	F2	F3	F4	F5
Quinate	37.4	34.8	38.8	41.4	43.2
Fumarate	32.8	29.1	34.4	36.6	33.1
Glutamate	17.9	16.4	14.8	13.9	14.7
Leucine	14.9	15.5	16.1	12.8	13.3
Isoleucine	17.7	17.1	18.6	14.4	15.9
Alanine	18.3	19.9	17.6	12.9	14.1

The cultivation profile of *C. glutamicum SL* on grass silage juice is shown in Figure 4.1. All major carbon sources were consumed simultaneously as depicted in Figure 4.1A and depleted according to their initial concentrations.

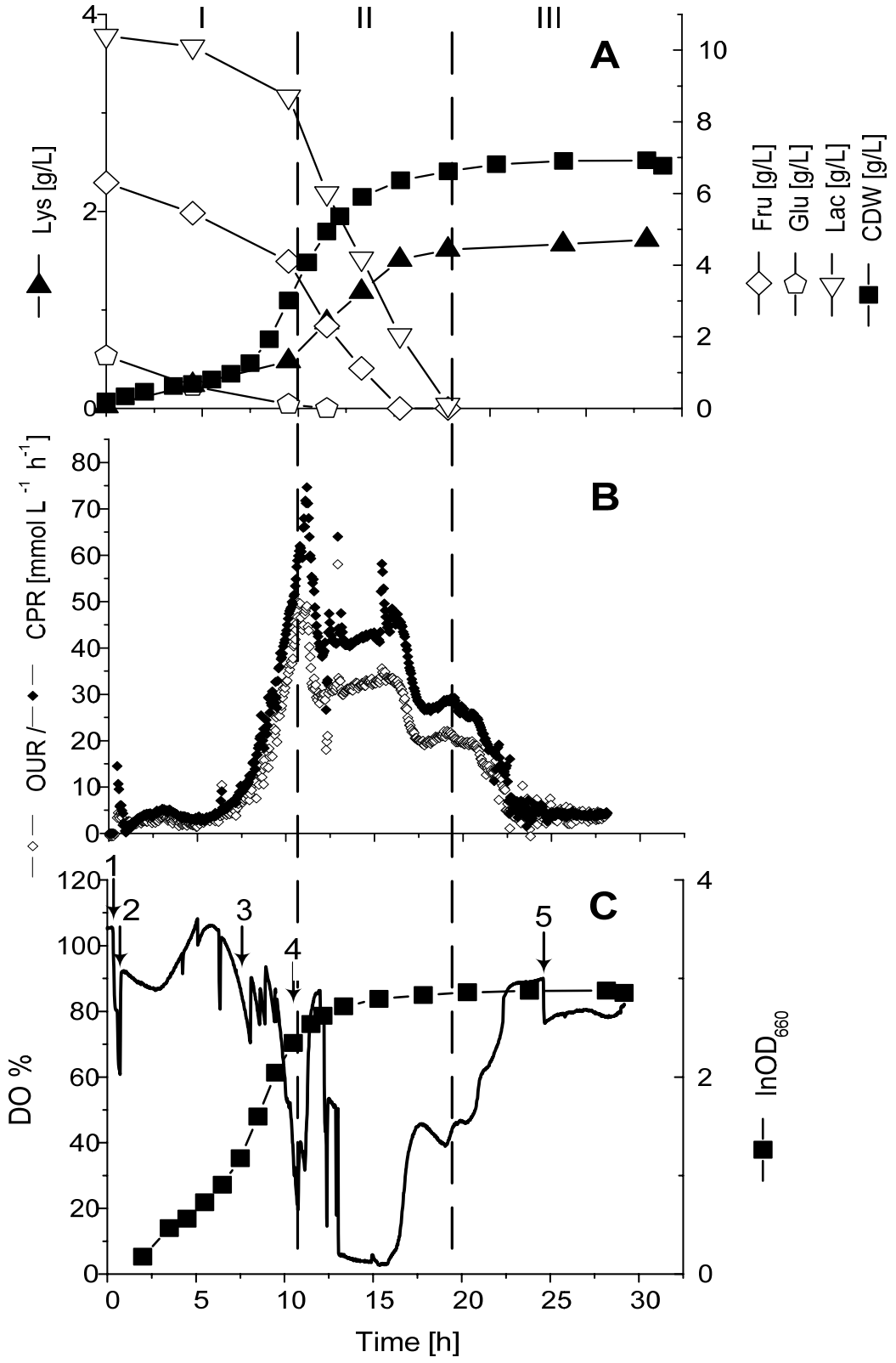


Fig. 4.1: Batch cultivation of L-lysine producing *C. glutamicum* $lysC^{fbr}$ dld_{Psod} $pyc_{Psod}$$malE_{Psod}$$fbp_{Psod}$$gapX_{Psod}$ (*C. glutamicum* SL) on 1:4 diluted grass silage juice in the bioreactor using impeller 1. (A) Concentrations of substrates fructose (Fru), glucose (Glu) and lactate (Lac), of product L-lysine (Lys) and of biomass provided as

cell dry weight (CDW). (B) Oxygen uptake rate (OUR) and carbon dioxide production rate (CPR). (C) Optical density (OD) and dissolved oxygen concentration (DO – full line) given in % air saturation and stirrer adjustment: 1 – 800 rpm, 2 – 1000 rpm, 3 – 1500 rpm, 4 – 2000 rpm, 5 – 1000 rpm. Dashed lines indicate identified growth phases I–III.

Glucose was exhausted at about 12 h when oxygen uptake rate (OUR) and carbon dioxide production rate (CPR) reached a distinct maximum value (Figure 4.1B). Simultaneously dissolved oxygen concentration (DO) increased rapidly. Fructose was depleted at about 17 h and the respiration rates reached another less pronounced maximum. After 18 h of cultivation, all carbon sources were completely consumed also indicated by another local maximum of respiration rates. Although grass silage was diluted 1:4 with water, the aeration capacity of the bioreactor applied was insufficient. At time points 1, 2, 3 and 4 depicted in Figure 4.1C the stirrer speed was stepwise increased up to the maximum possible value of 2000 rpm. Nevertheless, DO fell below 5% air saturation after 13 h. After depletion of fructose DO increased again. In this culture the growth in phase I was exponential with two different rates, 0.16 h^{-1} until about 7 h and 0.39 h^{-1} until about 10 h. L-lysine concentration increased steadily throughout the cultivation, reaching a final concentration of 1.7 g L^{-1}. The fermentation profile shows three distinct phases, mostly dependent on oxygen availability. In the first phase (0 h to 11 h), characterized by exponential growth under oxygen sufficient conditions, glucose, fructose and lactate were consumed simultaneously. L–lysine was secreted and accumulated in the culture to eventually reach 0.48 g L^{-1} when glucose was completely depleted. Characteristic for this first phase are non-limiting oxygen concentrations and exponential growth. OUR and CPR were increasing indicating a higher oxygen demand and CO_2 production by the increasing biomass. During the second phase (11 h to 18 h), oxygen limitation no longer allowed exponential growth. During this phase biomass formation slowed down while L-lysine secretion resulted in a concentration increase to 1.6 g L^{-1}. During this oxygen limited phase CPR was higher than OUR most probably due to oxygen limitation. The resulting average RQ-value was about 1.3 between 7 h to 22 h cultivation with large noise before and after that due to error amplification (Heinzle et al., 1990). This value can be explained by the use of lactate and other organic acids as carbon and energy sources and the higher degree of reductance of L-lysine compared to carbohydrates (Stephanopoulos et al., 1998). In the

third phase (18 h to 28 h), substrate consumption was complete and biomass and L-lysine reached final levels of 6.9 g L^{-1} and 1.7 g L^{-1}, respectively, resulting in 0.24 g L-lysine (g biomass)$^{-1}$. While dissolved oxygen levels increased, OUR and CPR decreased below 5 mmol L^{-1} h^{-1} remaining essentially constant after 23 hours. No significant byproduct formation could be observed in this phase. The produced amount of carbon dioxide was 461 mmol L^{-1}. Additionally accounting for produced biomass and L-lysine, a total carbon output of 810 mmol versus 743 mmol carbon input was determined. This means that the origin of only 8.3% of the carbon detected in products is not clear. The difference is actually within the experimental error of these measurements of around 5% each. The detected overproduction of carbon containing products may originate from other compounds originally contained in the silage that were not analytically detected but converted to biomass, L-lysine or CO_2.

Non-limiting conditions concerning dissolved oxygen (always above 60% DO) were achieved by gassing pure oxygen and using larger impellers, thus increasing the specific power input and therefore increasing oxygen transfer rate. The cultivation profile is depicted in Figure S 4.1. However, only slight differences could be observed compared to the oxygen limiting conditions. The L-lysine yield slightly increased to 0.28 g L-lysine (g biomass)$^{-1}$, and a lower RQ value of 1.15 was determined. In general, one can concluded that growth and L-lysine production seem to be quite independent on the varying oxygen supply in both cases and only minor changes could be observed, however, reaching a L-lysine carbon yield of 9.4% indicates the potential of further improvements based on well-known genetic modifications (Becker and Wittmann, 2012).

As depicted in Figure 4.2 cultivations on corn silage juice showed a very similar profile concerning biomass and product formation as well as substrate uptake characteristics and specific growth rate. In contrast to grass silage juice corn silage has a starch content of about 300 g L^{-1} that cannot be readily metabolized by *C. glutamicum* (Seibold et al., 2006; Tateno et al., 2007). After 5 hours, DO fell below 5% and exponential growth switched to linear growth until the exhaustion of major monomeric carbon sources. After 13 hours, the resulting concentration of L-lysine was 1.37 g L^{-1} and of biomass 6.1 g L^{-1} corresponding to 0.23 g L-lysine (g biomass)$^{-1}$, a value almost equal to the one of grass silage shown in Figure 4.1. Starch hydrolysis using amylases would allow increasing the amount of L-

lysine produced dramatically. In this case, a fed batch fermentation would most likely be optimal, since *C. glutamicum* would react to an excess of carbon supply with a likely increased byproduct formation as shown for the L-lysine producing strain *C. glutamicum* ATCC 21513 (Hadj Sassi et al., 1998). Despite the significant difference in silage composition the biomass yield was very similar to grass silage (Table 4.7) whereas L-lysine yield was slightly lower.

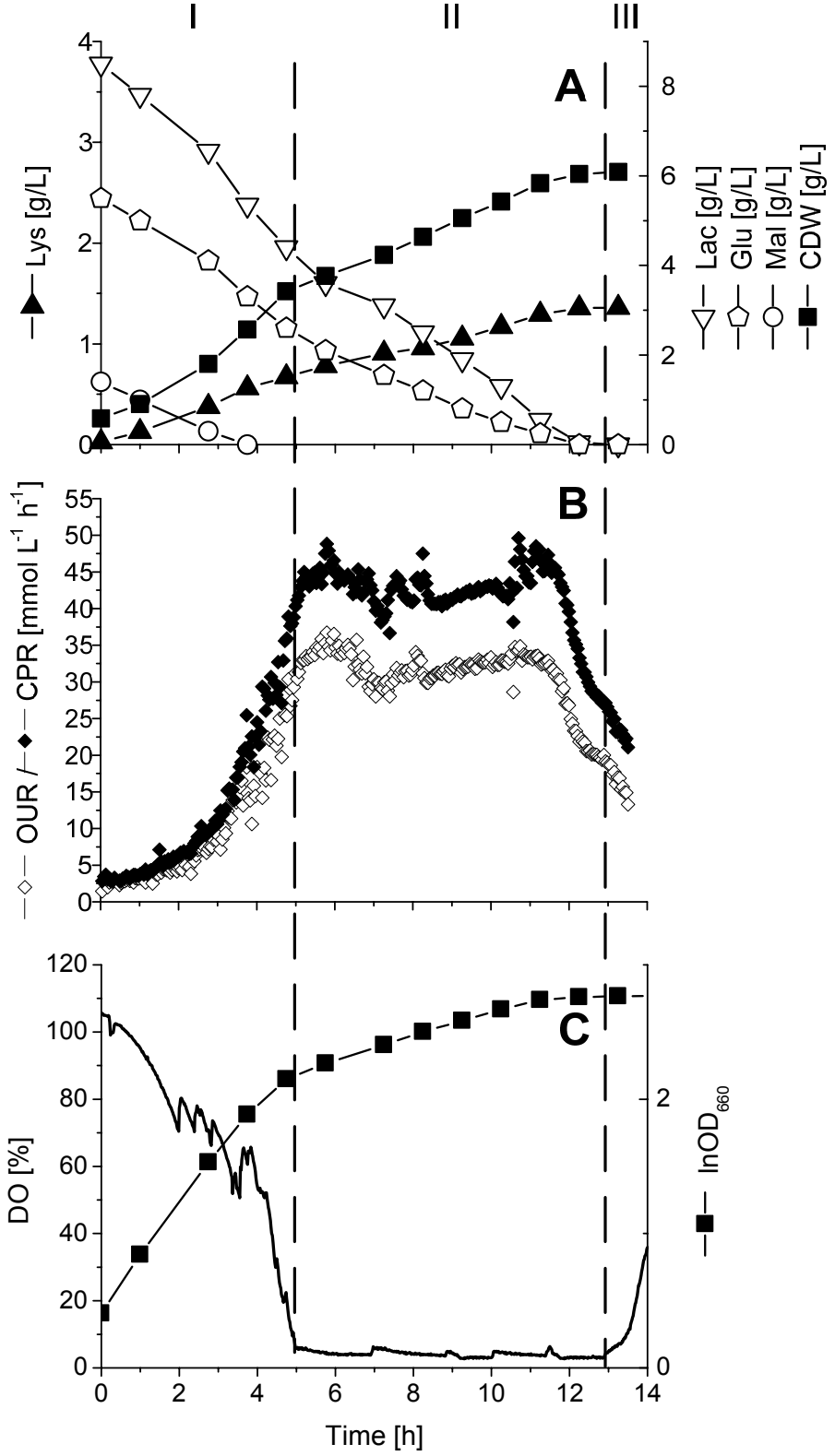


Fig. 4.2: Batch cultivation of lysine producing *C. glutamicum* SL on 1:4 diluted corn-silage juice in the bioreactor using impeller 1 with 800 rpm. (A) Concentrations of substrates maltose (Mal), glucose (Glu) and lactate (Lac), of product L-lysine (Lys)

and of biomass provided as cell dry weight (CDW). (B) Oxygen uptake rate (OUR) and carbon dioxide production rate (CPR). (C) Optical density (OD) and dissolved oxygen concentration (DO – full line) given in % air saturation and stirrer and oxygen adjustment. Dashed lines indicate identified growth phases I–III.

Shake flask cultivation using undiluted silage juice

To study the culture behavior with undiluted silage juice under oxygen limitation we applied shake flask cultivations. These cultivations were performed using flasks with fluorescence based dissolved oxygen sensing (Schneider et al., 2010). Both cultivations, on grass silage juice and on corn silage juice show two different phases – one non-limiting and one limiting concerning oxygen, as displayed in Figure S 4.2 and S 4.3. Non-limiting growth in shake flasks was comparable to non-limited growth observed in bioreactors for both silage juices. The same was observed for oxygen limiting conditions on both silage juices. Our developed strain *C. glutamicum* SL, did not show any reduction in specific growth rate or any other kind of lactic acid induced stress contrary to previous studies (Seletzky et al., 2006). Product and biomass formation as well as L-lysine yield in shake flasks were very similar on both types of silage (see Table 4.7). Even in comparison to bioreactor cultivations applying oxygen sufficient and limiting conditions comparable yields were obtained (Table 4.7). Compared to the precedent mutant strain *C. glutamicum* $lysC^{fbr}$ dld_{Psod} pyc_{Psod} $malE_{Psod}$, with a selectivity of 0.067 g L-lysine (g biomass)$^{-1}$, the L-lysine selectivity of the new strain increased about four times.

Connections between growth, substrate consumption and L-lysine production are summarized in concentration biomass plots of all cultivations shown in Figure 4.3. In all cases the substrates fructose, glucose, lactate and maltose were consumed simultaneously. The time point of depletion of the substrates was depending on their initial concentration. The selectivity for L-lysine as indicated by the slope of the L-lysine curve was nearly constant in both corn silage fermentations (Figure 4.3C and D) and in most parts of the shake flask grass silage cultivation (Figure 4.3B), whereas in the grass silage fermentation in the bioreactor depicted in Figure 4.1 it increased towards the end of cultivation (Figure 4.3A).

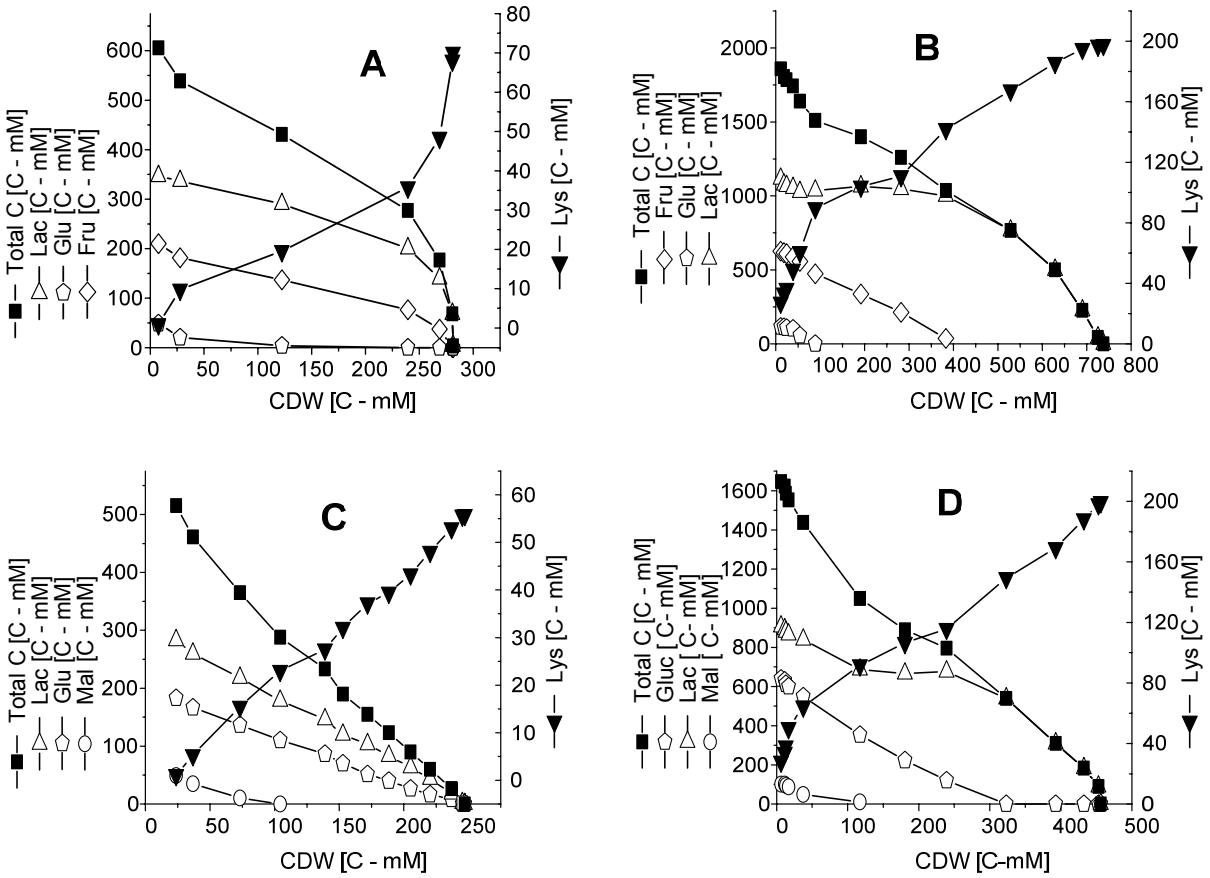


Fig. 4.3: Relation of substrate and product concentrations and biomass production in L-lysine producing *C. glutamicum* SL cultures on grass and corn silages. (A) Diluted grass silage with raw data shown in Fig. 4.1. (B) Undiluted grass silage cultivated in shake flasks (Figure S 4.2). (C) Diluted corn silage with raw data shown in Fig. 4.2. (D) Undiluted corn silage cultivated in shake flasks (Figure S 4.3). Concentrations of substrates fructose (Fru), maltose (Mal), glucose (Glu) and lactate (Lac), of product L-lysine (Lys) and of biomass provided as cell dry weight (CDW). Total carbon (total C) comprises only those substrates shown in the respective part of the figure.

Despite the differences of the silage juices regarding available carbon sources, the created mutant strain *C. glutamicum SL* showed robust and flexible production of L-lysine. It was

growing fast, with an almost constant L-lysine selectivity of 0.25 ± 0.03 g L-lysine (g biomass)$^{-1}$ and L-lysine carbon yields of slightly below 10% tolerating substantial changes in the profile of available carbon sources, cultivation methods and oxygen supply. This is very important, since the carbon source composition in silage juices is strongly varying, depending on factors like the used green biomass, ensiling technique and conditions as well as effects of wilting and drying (Hirst and Ramstad, 1957). With a secreted L-lysine concentration of 4.9 g L^{-1} and additionally 0.5 g L^{-1} contained in the cellular biomass, estimated from 17 g L^{-1} using data of Wittmann and de Graaf (2005), the demanded L-lysine supplementation of 0.4% of total protein to optimize the nutritional value of silage would be easily achievable. A comparison between the used silages with varying composition and the performance of the strains under the various cultivation conditions is shown in Table 4.7.

Table 4.7: Comparison of the used silage juices and the performance of the strains under the various cultivation conditions.

	Main Carbon Sources [g L^{-1}]						$Y_{P/S}$	$Y_{X/S}$	n
	Glucose	**Fructose**	**Maltose**	**Lactic acid**		**μ [h^{-1}]**	**[C-mmol (C-mol)$^{-1}$]**	**[C-mmol (C-mol)$^{-1}$]**	
Grass	4.0 ± 2.0	20.0 ± 4.0	-	40.0 ± 7.5	**BR**	0.36 ± 0.01	94 ± 2	380 ± 8	3
					SF	0.35 ± 0.01	86 ± 3	340 ± 6	2
Corn	2.0 ± 1.5	-	1.5 ± 0.5	30.0 ± 2.0	**BR**	0.35 ± 0.01	87 ± 2	385± 9	2
					SF	0.35 ± 0.01	99 ± 3	362 ± 8	2

BR – bioreactor, SF – shake flasks, μ – specific growth rate, $Y_{P/S}$ – carbon yield of L-lysine on substrates, carbon yield of L-lysine on substrates, $Y_{X/S}$ – carbon yield of biomass on substrates. n – number of experiments. Carbon yields are calculated considering all substrates measured, glucose, fructose, maltose, lactic acid as depicted in Figure 4.3 as well as quinate, fumarate, glutamate, alanine, leucine and isoleucine listed in Table 4.6.

This work indicates the robustness of the engineered organism *C. glutamicum* SL being able to adapt to varying oxygen availabilities without major drawbacks concerning the L-lysine producing ability, indicated by almost constant L-lysine yields under the varying oxygen conditions investigated. This also holds for different silage juices obtained from different sources which varied significantly in their composition.

Concluding remarks

Sustainable economic growth requires safe, sustainable resources for industrial productions as well as innovative ideas and technologies. Large scale ensiling of green biomass for the production of methane opens up new substrate opportunities for biotechnological production and assures a supply throughout the year, independent on sugar market price. In this regard, the present work describes the engineering of *C. glutamicum* into efficient, fast growing L-lysine producers using silage juice as a novel, renewable substrate. An already created mutant (Neuner and Heinzle, 2011) seemed highly suitable for further engineering. The performed pathway analysis using the network model and analysis method of Neuner and Heinzle (Neuner and Heinzle, 2011) with inclusion of fructose as additional substrate allowed to identify two additional targets, fructose 1,6-bisphosphatase and glyceraldehyde 3-phosphate. The first was already shown to increase L-lysine yield by increasing NADPH availability and reducing byproduct formation. The overexpression of *gapX* caused a reduction of formation of the byproduct dihydroxyacetone. The resulting strain, *C.glutamicum* SL was stable and fast growing. It clearly showed an improved performance compared to the precedent strain exhibiting a threefold increased L-lysine yield and a drastic reduction of dihydroxyacetone formation. These characteristics make the *C. glutamicum* $lysC^{fbr}$ dld_{Psod} pyc_{Psod} $malE_{Psod}$ fbp_{Psod} $gapX_{Psod}$ mutant a very versatile, adaptive strain and show the potential of silage as a renewable, sustainable substrate for biotechnological applications. The obtained carbon yields of 86 C-mmol (C-mol)$^{-1}$ to 94 C-mmol (C-mol)$^{-1}$ are below those reported in the literature for high producing strains but provide a good starting point for extended engineering using further well known genetic modifications (Becker and Wittmann, 2011; Becker and Wittmann, 2012; Becker et al., 2011). Future work regarding L-lysine production on silage is directed towards further increase of L-lysine yield, selectivity and product titer. L-lysine production was hardly effected by low oxygen supply. This is very interesting for an efficient production creating reduced aeration cost both in terms of investment as well as operation costs. Summing up, this work shows that silages are promising raw materials for biotechnological production using *C. glutamicum*.

Acknowledgements. We thank the Fachagentur für Nachwachsende Rohstoffe (FNR) who supported this work (FKZ 22004908). KS acknowledges support by BMBF (Federal Ministry of Education and Research - Germany, Project SWEEPRO, FKZ 0315800B). We are very grateful for the most valuable support by Michel Fritz concerning instrumental analysis.

Chapter 5

Pretreatment of corn silage juice

Corn silage contains considerable amounts of starch (~300 g L^{-1}), the major storage polysaccharide of cereal grains. Starch is a substrate not metabolizable by *C. glutamicum* wilde type due to the absence of starch degrading enzymes (Seibold et al., 2006; Tateno et al., 2007). In order to assess the possibilities for starch utilization, we investigated special pretreatment methods for corn silage juice. A schematic overview is depicted in Figure 5.1.

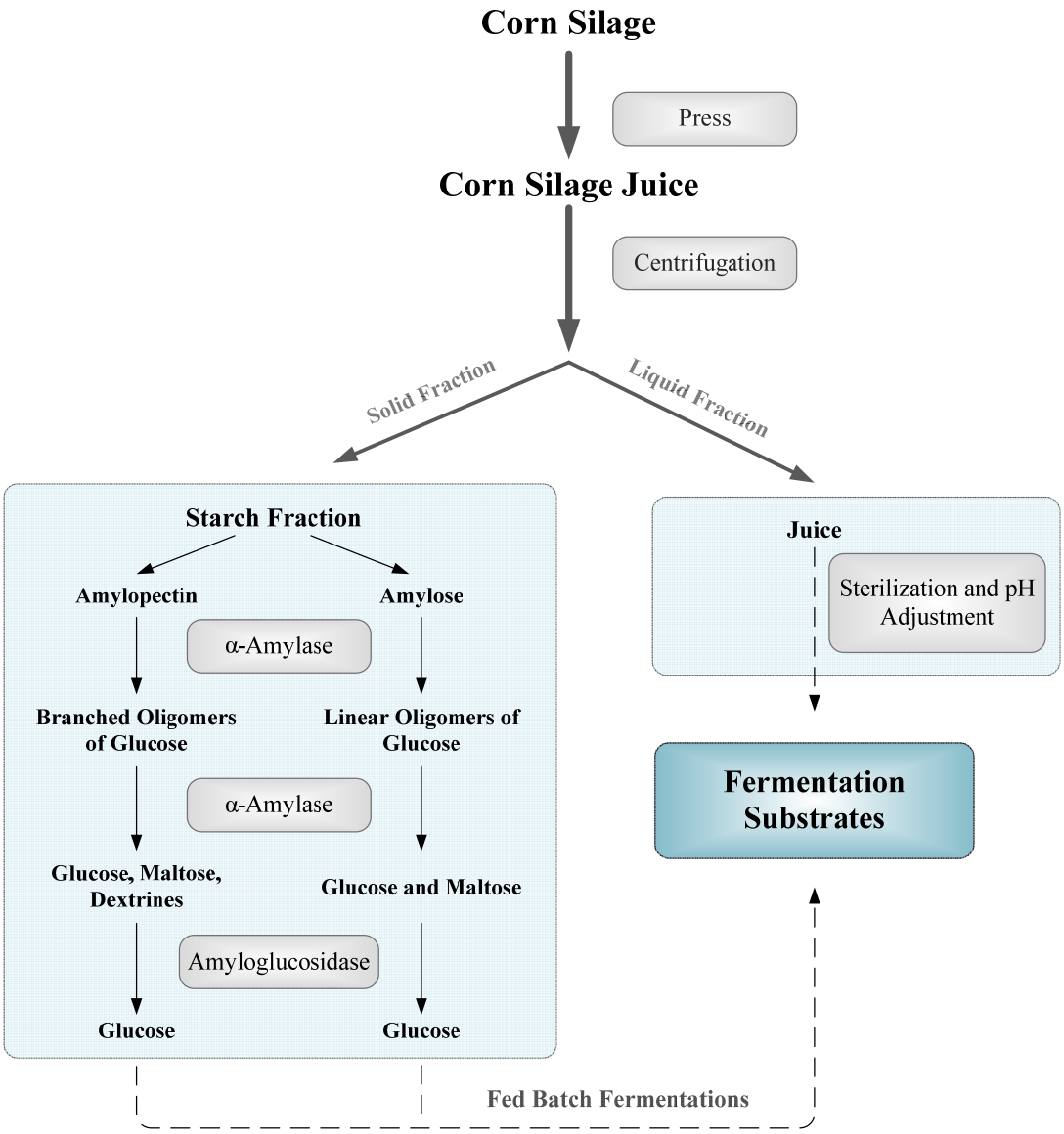
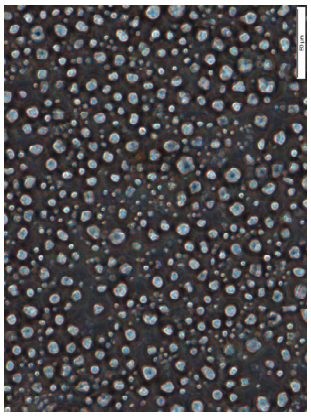
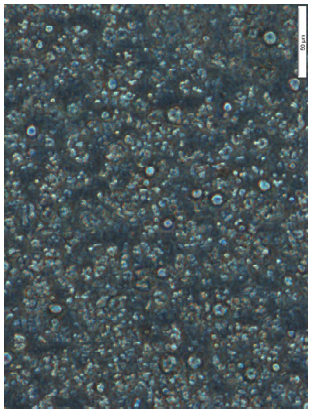
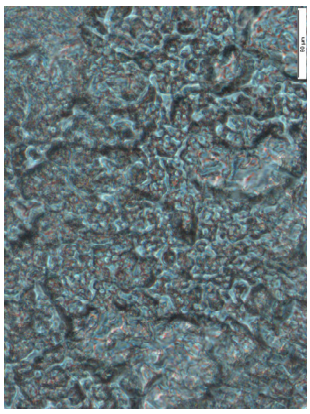


Fig. 5.1: Schematic overview of the corn silage pretreatment. Pretreatment steps are highlighted in grey boxes.

Enzymatic starch hydrolysation of the pressed silage juice using α-amylase from *Aspergillus oryzae* (Sigma, Switzerland) and amyloglucosidase from *Aspergillus niger* (Sigma, Switzerland) reduced the size of the starch grains and degraded the available

starch to maltose and glucose. During the procedure, glucose and maltose concentrations were determined using HPLC. The morphology of the starch grains was documented using an IX70 microscope (Olympus, Hamburg, Germany).The results are shown in Table 5.1.

Table 5.1: Pretreatment of corn silage juice. Starch hydrolysis using α-amylase and amyloglucosidase.

	No Pretreatment	**α-Amylase-Treatment**	**Amyloglucosidase-Treatment**
Morphology			
Concentration [g L^{-1}]			
Maltose	0	40.1	10.9
Glucose	0	8.7	151.1

During the initial phase, starch grains are clearly visible and no glucose or maltose is detectable. After the use of α-amylase for 40 h, the size of the starch grains is significantly altered, the grains are smaller and a maltose concentration of 40.1 g L^{-1} was detected, with a glucose concentration of 8.7 g L^{-1}. The analysis of the sample after the use of amyloglucosidase for another 40 h clearly shows the influence of the enzyme regarding starch grain morphology and the concentrations of glucose and maltose. The grains are barely visible, with a maltose concentration of 10.9 g L^{-1} and 151 g L^{-1} glucose in the supernatant. Since the initial starch concentration in the used corn silage juice was 214 g L^{-1}, the enzymatic treatment converted 75% of the starch into maltose and glucose, making the carbon available for *C. glutamicum*. This is very beneficial for two different reasons. First, there is a lot more carbon available for L-lysine fermentations. Second, when starch supplied from corn silage is fed to animals, incomplete digestion of non-fiber polysaccharides like starch appears to be very common for lactating dairy cows and other ruminants (Eastridge et al., 2011). Rapid fermentation of starch in the rumen increases the acidity in this compartment and the risk of digestive disorders, particularly ruminal acidosis (Johnson et al., 2003). An enzymatic pretreatment would convert a high percentage of the carbon from starch to substrates available for *C. glutamicum* that can be converted to L-lysine. At the same time, the final starch concentration in the fodder is reduced and the incomplete digestion, where around 25% of the energy contained in starch would be lost (Huntington, 1997), can be avoided. This would significantly improve the economic efficiency of the process. For all the cultivation experiments performed with corn silage juice, the starch fraction has not been used.

Chapter 6

Concluding discussion and remarks

Concluding discussion

The depletion of fossil resources together with a constantly growing population and environmental issues like global warming and increasing waste problems call for sustainable alternatives regarding energy, fuels and chemical production. Solar, wind and water energy are already exploited as alternatives, but can only partially replace the fossil energy. For the production of transportation fuels and chemicals, the utilization of plant biomass shows the greatest potential. Until now, renewable raw materials had an economic disadvantage, especially if it comes to the production of bulk products, as they are priced higher than fossil resources, compared to their respective product yield, sophisticated logistics due to low transport densities and short shelf life. These facts impeded the development of bio-based processes, leaving fossil raw materials the undisputed primacy as an industrial feedstock. However, expected price increases of crude oil and the limited availability of these resources are profoundly challenging this primacy. As a result of this development, new technologies based on plant biomass, aiming at multi-product plants with increased efficiency of biomass utilization arised. Using low priced materials and utilizing these resources to the utmost possible degree will eventually compensate for the initial economic disadvantage of bio-based feedstocks, allowing these technologies to compete with conventional processes based on fossil resources. It is obvious that technical and economic barriers must be conquered for an efficient utilization of these feedstocks. Many of the economic problems will be solved, when the technical issues are resolved. Additionally, the price increase of petroleum based resources will also contribute to the economic feasibility of bio-based products.

In this thesis, the potential use of silage and silage juice for the biotechnological production of the amino acid L-lysine was investigated. Three different aspects regarding amino acid production on silage have been addressed. First, in order to make a bio-based process efficient, the substrate spectrum, anabolic power supply in form of NADPH and precursor availability were optimized. By performing a metabolic network analysis, specific targets for genetic engineering were identified. Second, the treatment of silage juice for optimal use and the influence of process parameters like aeration and stirring rate on production efficiency were elucidated. Finally, L-lysine production on different silage juices was adressed.

Expansion of the substrate spectrum and L-lysine production on synthetic silage juice

Two major compounds of silage juice are lactate and glucose. It was of interest to investigate the use of such a type of raw material, consisting of considerable amounts of lactate and glucose, for the biotechnological production of L-lysine. Growth of *C. glutamicum* on mixtures of glucose and racemic lactate has not been studied in detail before and attempts to overproduce L-lysine have not been reported yet using such substrates. *C. glutamicum* wild type shows poor growth on D-lactate and racemic lactate mixtures (Scheer et al., 1988). As thoroughly described in **Chapter 3,** overexpression of the constitutively expressed D-lactate dehydrogenase (*dld*) expanded the substrate spectrum and improved growth. A production strain capable of completely metabolizing carbon sources contained in silage juice would be desirable, in order to increase the carbon utilization of a production host. The engineered strain is currently able to utilize D-lactate in addition to its usual carbon sources, resulting in an increased carbon efficiency and fast growth. The overexpression of *dld* significantly improved growth on racemic lactate with respect to growth rate (55% increase compared to parental strain) as well as biomass yield (17% increase). The increase in growth rate is more than could be expected from a simple duplication of lactate supply by *dld* overexpression and represents a good starting point for further metabolic engineering. Although this strain shows no detectable, growth coupled L-lysine production, it can be further optimized regarding NADPH supply and precursor availability for L-lysine production. By implementing a network analysis, the maximum theoretical yield of L-lysine on glucose and lactate mixtures was calculated based on the stoichiometry of the metabolic network of *C. glutamicum*. The highest L-lysine carbon yield of an elementary flux mode with simultaneous consumption of glucose and lactate was 84.4%. However, this is a so called extreme mode, producing exclusively L-lysine, with no biomass formation. Regarding modes with simultaneous glucose and lactate consumption as well as simultaneous biomass and L-lysine biosynthesis, the maximum theoretical carbon yield is 53% with an associated biomass carbon yield of 19%. The elementary flux mode analysis combined with useful objective functions (see **Chapter 3**) permitted a valuable prediction of potential targets for increasing L-lysine production by overexpression of selected genes. This analysis predicted stimulation of L-lysine

production by a combined overexpression of pyruvate carboxylase and malic enzyme as the most promising targets, followed by gluconeogenic enzymes and enzymes of the pentose phosphate pathway. Compared to the parental strain, *C. glutamicum* $lysC^{fbr}$, exhibiting a growth rate of 0.13 h^{-1} on glucose-lactate mixtures, the sole overexpression of D-lactate dehydrogenase (*dld*) in *C. glutamicum* $lysC^{fbr}dld_{Psod}$ increased the growth rate by more than 100% (μ = 0.28 h^{-1}). The additional overexpression of pyruvate carboxylase (*pyc*) further increased the growth rate up to 0.39 h^{-1}.One possible reason why the parental strain *C. glutamicum* $lysC^{fbr}$ did not produce detectable amounts of L-lysine neither on lactate, nor on glucose-lactate mixtures, seems to be the availability of NADPH. A fact supporting this interpretation is that lactate uptake increased after the overexpression of D-lactate dehydrogenase (*dld*), but still no L-lysine was produced. Overexpression of pyruvate carboxylase (*pyc*) and malic enzyme (*malE*) enabled L-lysine production on synthetic silage juice, by increasing the precursor supply in form of oxaloacetate and providing enough NADPH for biomass and L-lysine production. The resulting strain *C. glutamicum* $lysC^{fbr}dld_{Psod}pyc_{Psod}malE_{Psod}$, with a selectivity of 0.067 g L-lysine (g biomass)$^{-1}$ when cultivated on silage juice is a promising target for further engineering. The positive effects facilitated by the modified gene expression regarding growth and L-lysine production on synthetic silage juice prove the usefulness of combining bioinformatic methods with wet lab experiments.

L-lysine production on silage juices

In **Chapter 4**, the application of silage juice for biotechnological use has been investigated. Sterilization of the silage juice and the avoidance of inhibitory compounds due to the occurring Maillard reaction are a crucial step for the use of silage juice as a fermentation substrate. Different pretreatment methods for grass and corn silage have been discussed, according to the respective silage juice characteristics. Regarding corn silage juice, the high amount of starch (~ 300 g L^{-1}) is not available for *C. glutamicum*, since it is not able to directly metabolize starch. The starch represents a substantial amount of carbon and it is of major interest to investigate ways to channel it into L-lysine production, as described in **Chapter 5**. With a maltose concentration of 10.9 g L^{-1} and 151 g L^{-1} glucose

in the supernatant after enzymatic treatment, valuable carbon sources became readily available. These findings open up new possibilities for biotechnological use. The high glucose concentration would be suitable for fed batch cultivation, providing a good perspective for L-lysine production. The downside is the more sophisticated fermentation equipment and the impossibility of a simple fermentation that can be performed directly on farming units as well as the economic factor. The additional use of enzymes is a cost factor that must be taken into account. A different option would be co-fermentation using microorganisms secreting starch degrading enzymes or even the use of engineered *C. glutamicum* strains, displaying α-amylase from e.g *Streptococcus bovis* on its cell surface (Tateno et al., 2007). Heterologuous gene expression would imply that the constructed strain belongs to the GMO category. This means that the use of L-lysine as a feed supplement would no longer be an option. In this context, heterologuous expression of genes involved in pentose metabolism (xylose) can also be mentioned. Silage contains xylose, in amounts between 5-8 g L^{-1}, depending on the used biomass. Xylose cannot be metabolized by *C. glutamicum* wild type (Kawaguchi et al., 2006). This genetic modification would increase the amount of available carbon, at the same time excluding the optional use of L-lysine as a feed supplement under the prevailing conditions for acceptance of GMO's. Besides the investigation of proper methods for silage juice treatment, the strains described in **Chapter 3** have been further optimized towards L-lysine production, improved growth as well as reduced byproduct formation and tested on grass and corn silage juices. Two additional targets for overexpression (*fbp* and *gapX*) that were predicted by the same model and method used in **Chapter 3**, but using lactate, glucose and fructose as substrates, were investigated. Overexpression of the fructose 1,6-bisphosphatase significantly improved the L-lysine yield on grass and corn silage juice, while the byproduct formation was reduced. Additional overexpression of glyceraldehyde 3-phosphate dehydrogenase further diminished the byproduct formation. Keeping in mind that one of the top challenges regarding bio-based products is the economic production, different cultivation setups have been tested, in order to assess the influence and necessity of aeration and oxygen supply during the fermentation. All these factors contribute to the estimation of capital and operating costs, determining the feasibility of the process (Heinzle et al., 2006). *C. glutamicum SL* performed well under the tested cultivation setups, not showing any reduction in specific growth rate or any other kind of lactic acid induced stress due to high concentrations in undiluted silage juices. Product and biomass formation as well as L-lysine yield in shake flasks were very similar on both types of

silage. Even in comparison to bioreactor cultivations applying oxygen sufficient and limiting conditions comparable yields were obtained, showing the robustness of the created strain. It can adapt to varying oxygen availabilities without major drawbacks concerning the L-lysine producing ability, indicated by almost constant L-lysine yields under the varying oxygen conditions investigated. A detailed description and discussion of these findings is available in **Chapter 4**. This also holds for the productivity on different silage juices which varied significantly in their carbon source composition. The obtained L-lysine carbon yields of 86–94 C-mmol (C-mol)$^{-1}$ and a L-lysine selectivity of 0.25 ± 0.03 g L-lysine (g biomass)$^{-1}$ at growth rates of 0.35 ± 0.01 h^{-1} are a promising starting point for extended engineering. All performed genetic modifications are genome based, without the need of any selectivity markers like antibiotic resistance remaining in the strains. This makes the *C. glutamicum SL* strain suitable and very interesting candidate for the feed and farming industry, where L-lysine supplementation is of major interest. This fact and the GRAS classification of *C. glutamicum* allow the use of the complete fermentation broth, containing the microorganisms, as a feed supplement. The mentioned fodder supplementation with 0.4% L-lysine (as described in **Chapter 4**) in order to increase the PER of corn protein is already achieved. This is a considerable advantage compared to conventional processes for crystalline L-lysine*HCl production where the accumulated biomass, byproducts and residues from the fermentation process must be disposed. Purification of the L-lysine using ion exchange chromatography is also associated with a loss of considerable amounts of the product. Conventional amino acid production is a costly process, requiring the handling of hazardous substances with a high ecological burden like ammonia solution as an eluent for the ion exchange columns and hydrochloric acid for the neutralization of the L-lysine base (Kelle et al., 2005). The idea presented here describes a simpler route to feed supplementation which avoids the ecological and technical burden of existing methods. Besides that, L-lysine is available as a natural, free amino acid with a high biological efficacy, produced exclusively from renewable resources without any additive.

Concluding remarks

This thesis investigates the use of silage and silage juice as renewable fermentation substrates for L-lysine production with *C. glutamicum*. In particular, we showed the great potential of L-lysine overproduction as predicted by the performed metabolic network analysis. The determination of the maximal theoretical yields is very promising, since it is the evaluation of the feasibility of such a process. The performed elementary mode analysis was very useful for the identification of potential targets for genetic engineering, as demonstrated by the implementation of the findings in **Chapters 3** and **4**. A widened substrate spectrum and the associated improved carbon efficiency and growth characteristics were obtained. It became obvious, that a crucial step in L-lysine overproduction, especially on substrates employing gluconeogenic pathways, is the supply of anabolic reduction power in form of NADPH. The improved NADPH availability due to the overexpression of malic enzyme was proven to be of utmost importance, directly resulting in improved L-lysine yields. The constructed strains were further engineered towards better L-lysine selectivity, with increased L-lysine yields and a lower byproduct formation. A very important part of the thesis was a substrate oriented approach, where the chosen substrates, grass and corn silage juice, were tested for optimal use as an additive free, natural fermentation substrates. A new and innovative process for the biotechnological production of L-lysine was tested, where the whole manufacturing concept including the production strain, used raw materials and cultivation setups were systematically tailored for optimal environmental compatibility, reduced handling of hazardous materials as well as minimal resource depletion and waste generation. With a reduced investment outlay and being easily applicable on agricultural and farming units, the L-lysine fermentation on silage juices is highly attractive, in ecological and economic terms. By increasing the nutritional value of fodder, L-lysine fermentations on silage juices are a very promising, cost effective contribution to a sustainable economy, more environmentally compatible by the reduced nitrogen emission levels.

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Supplementary material

Supplementary material Chapter 3

Supplementary Tables

Table S 3.1: List of reactions used for elementary mode analysis (Figure 3.1). Single arrows – irreversible reactions, double arrows – reversible reactions. Formulation as used as input for efmtool (http:/www.csb.ethz.ch/tools/efmtool; (Terzer and Stelling, 2008). Reactions are deduced from (Krömer et al., 2004), KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/), (Kjeldsen and Nielsen, 2009; Melzer et al., 2009; Shinfuku et al., 2009).

R1 : # --> GLCex
R2 : # --> LACex
R3 : # --> O2
R4 : # --> NH3
R5 : NH3 --> #
R6 : # --> CO2
R7 : CO2 --> #
R8 : PEP + GLCex --> PYR + G6P
R9 : LACex + NAD --> PYR + NADH
R10 : G6P <--> F6P
R11 : G6P + NADP --> GLC-LAC + NADPH
R12 : GLC-LAC --> 6-P-Gluconate
R13 : 6-P-Gluconate + NADP --> RIB-5P + CO2 + NADPH
R14 : RIB-5P <--> XYL-5P
R15 : RIB-5P <--> RIBO-5P
R16 : S7P + GA3P <--> RIBO-5P + XYL-5P
R17 : S7P + GA3P <--> E-4P + F6P
R18 : F6P + GA3P <--> E-4P + XYL-5P
R19 : ATP + F6P --> ADP + F-16-BP
R20 : F-16-BP --> F6P
R21 : F-16-BP <--> GA3P + DAHP
R22 : DAHP <--> GA3P
R23 : GA3P + NAD <--> 13-PG + NADH
R24 : ADP + 13-PG <--> ATP + 3-PG

R25 : 3-PG <--> 2-PG
R26 : 2-PG <--> PEP
R27 : PEP + ADP --> PYR + ATP
R28 : PYR + H-CoA + NAD --> AC-CoA + NADH + CO2
R29 : AC-CoA + OAA --> CIT + H-CoA
R30 : CIT <--> Cis-ACO
R31 : Cis-ACO <--> ICI
R32 : ICI + NADP --> 2-OXO + CO2 + NADPH
R33 : 2-OXO + NH3 + NADPH --> GLU + NADP
R34 : 2-OXO + NAD + H-CoA --> SUCC-CoA + NADH + CO2
R35 : SUCC-CoA + ADP <--> SUCC + H-CoA + ATP
R36 : SUCC + FAD <--> FUM + FADH
R37 : FUM <--> MAL
R38 : MAL + NAD <--> OAA + NADH
R39 : ICI --> GLYOXY + SUCC
R40 : GLYOXY + AC-CoA --> MAL + H-CoA
R41 : PYR + ATP + CO2 --> OAA + ADP
R42 : PEP + CO2 --> OAA
R43 : OAA + ATP <--> PEP + ADP + CO2
R44 : OAA + GLU <--> ASP + 2-OXO
R45 : ASP + ATP --> ASP-P + ADP
R46 : ASP-P + NADPH --> ASP-SA + NADP
R47 : ATP --> ADP
R48 : MAL + NADP <--> PYR + CO2 + NADPH
R49 : 2 NADH + O2 + 4 ADP --> 2 NAD + 4 ATP
R50 : 2 FADH + O2 + 2 ADP --> 2 FAD + 2 ATP
R51 : 6231 NH3 + 205 G6P + 308 F6P + 879 RIBO-5P + 268 E-4P + 129 GA3P + 1295 3-PG + 652 PEP + 2604 PYR + 3177 AC-CoA + 1680 OAA + 1224 2-OXO + 16429 NADPH + 17002 ATP + 3111 NAD --> 40552 BIOMASS + 16429 NADP + 3177 H-CoA + 1227 CO2 + 17002 ADP + 3111 NADH
R52 : AMP + ATP --> 2 ADP
R53 : ASP-SA + PYR --> DHP
R54 : DHP + NADPH <--> NADP + PDC
R55 : PDC + NH3 + NADPH <--> DMP + NADP
R56 : PDC + SUCC-CoA --> SAK + H-CoA
R57 : SAK + GLU <--> SDP + 2-OXO
R58 : SDP --> DMP + SUCC
R59 : DMP --> LYS + CO2
R60 : GLCex + ATP --> G6P + ADP
R61 : LYS --> #
R62 : # --> AMP
R63 : BIOMASS --> #

Table S 3.2: Examples of elementary flux modes with highest L-lysine yields during growth on lactate (only fluxes with non-zero values are listed). A – mode with highest yield, B – mode with highest yield not using glucose 6-phosphate dehydrogenase.

A) Mode with highest yield. $Y_{Lys/S}$ =0.750 (C-mol L-lysine)/(C-mol lactate)

R# : Flux reaction
R2 : 4.0 # --> LACex
R3 : 1.5 # --> O2
R4 : 3.0 # --> NH3
R7 : 3.0 CO2 --> #
R9 : 4.0 LACex + NAD --> PYR + NADH
R10 : -3.0 G6P <--> F6P
R11 : 3.0 G6P + NADP --> GLC-LAC + NADPH
R12 : 3.0 GLC-LAC --> 6-P-Gluconate
R13 : 3.0 6-P-Gluconate + NADP --> RIB-5P + CO2 + NADPH
R14 : 2.0 RIB-5P <--> XYL-5P
R15 : 1.0 RIB-5P <--> RIBO-5P
R16 : -1.0 S7P + GA3P <--> RIBO-5P + XYL-5P
R17 : 1.0 S7P + GA3P <--> E-4P + F6P
R18 : -1.0 F6P + GA3P <--> E-4P + XYL-5P
R20 : 1.0 F-16-BP --> F6P
R21 : -1.0 F-16-BP <--> GA3P + DAHP
R22 : -1.0 DAHP <--> GA3P
R23 : -1.0 GA3P + NAD <--> 13-PG + NADH
R24 : -1.0 ADP + 13-PG <--> ATP + 3-PG
R25 : -1.0 3-PG <--> 2-PG
R26 : -1.0 2-PG <--> PEP
R33 : 1.5 2-OXO + NH3 + NADPH --> GLU + NADP
R41 : 2.5 PYR + ATP + CO2 --> OAA + ADP
R43 : 1.0 OAA + ATP <--> PEP + ADP + CO2
R44 : 1.5 OAA + GLU <--> ASP + 2-OXO
R45 : 1.5 ASP + ATP --> ASP-P + ADP
R46 : 1.5 ASP-P + NADPH --> ASP-SA + NADP
R49 : 1.5 2 NADH + O2 + 4 ADP --> 2 NAD + 4 ATP
R53 : 1.5 ASP-SA + PYR --> DHP
R54 : 1.5 DHP + NADPH <--> NADP + PDC
R55 : 1.5 PDC + NH3 + NADPH <--> DMP + NADP
R59 : 1.5 DMP --> LYS + CO2
R61 : 1.5 LYS --> #

B) Mode with highest yield not using glucose-6-phosphate dehydrogenase (R11). $Y_{Lys/S}$ =0.722 (C-mol L-lysine)/(C-mol lactate)

R# : Flux reaction
R2 : 7.2 # --> LACex
R3 : 3.4 # --> O2
R4 : 5.2 # --> NH3
R7 : 6.0 CO2 --> #
R9 : 7.2 LACex + NAD --> PYR + NADH
R28 : 2.0 PYR + H-CoA + NAD --> AC-CoA + NADH + CO2
R29 : 2.0 AC-CoA + OAA --> CIT + H-CoA
R30 : 2.0 CIT <--> Cis-ACO
R31 : 2.0 Cis-ACO <--> ICI
R32 : 2.0 ICI + NADP --> 2-OXO + CO2 + NADPH
R33 : 2.6 2-OXO + NH3 + NADPH --> GLU + NADP
R34 : 2.0 2-OXO + NAD + H-CoA --> SUCC-CoA + NADH + CO2
R35 : 2.0 SUCC-CoA + ADP <--> SUCC + H-CoA + ATP
R36 : 2.0 SUCC + FAD <--> FUM + FADH
R37 : 2.0 FUM <--> MAL
R38 : -6.4 MAL + NAD <--> OAA + NADH
R41 : 11.0 PYR + ATP + CO2 --> OAA + ADP
R44 : 2.6 OAA + GLU <--> ASP + 2-OXO
R45 : 2.6 ASP + ATP --> ASP-P + ADP
R46 : 2.6 ASP-P + NADPH --> ASP-SA + NADP
R48 : 8.4 MAL + NADP <--> PYR + CO2 + NADPH
R49 : 2.4 2 NADH + O2 + 4 ADP --> 2 NAD + 4 ATP
R50 : 1.0 2 FADH + O2 + 2 ADP --> 2 FAD + 2 ATP
R53 : 2.6 ASP-SA + PYR --> DHP
R54 : 2.6 DHP + NADPH <--> NADP + PDC
R55 : 2.6 PDC + NH3 + NADPH <--> DMP + NADP
R59 : 2.6 DMP --> LYS + CO2
R61 : 2.6 LYS --> #

Table S 3.3: Examples of elementary flux modes with highest L-lysine yields during growth on glucose (only fluxes with non-zero values are listed). A – mode with highest yield including malic enzyme activity, B – mode with highest L-lysine yield including malic enzyme activity and pts but without glucokinase, C – mode with highest yield excluding malic enzyme activity and using only pts.

A) mode with highest yield including malic enzyme activity (R48). $Y_{Lys/S}$ =0.857 (C-mol L-lysine)/(C-mol glucose)

R# : Flux reaction

R1 : 7 # --> GLCex
R4 : 12 # --> NH3
R7 : 6 CO2 --> #
R10 : 1 G6P <--> F6P
R11 : 6 G6P + NADP --> GLC-LAC + NADPH
R12 : 6 GLC-LAC --> 6-P-Gluconate
R13 : 6 6-P-Gluconate + NADP --> RIB-5P + CO2 + NADPH
R14 : 4 RIB-5P <--> XYL-5P
R15 : 2 RIB-5P <--> RIBO-5P
R16 : -2 S7P + GA3P <--> RIBO-5P + XYL-5P
R17 : 2 S7P + GA3P <--> E-4P + F6P
R18 : -2 F6P + GA3P <--> E-4P + XYL-5P
R19 : 5 ATP + F6P --> ADP + F-16-BP
R21 : 5 F-16-BP <--> GA3P + DAHP
R22 : 5 DAHP <--> GA3P
R23 : 12 GA3P + NAD <--> 13-PG + NADH
R24 : 12 ADP + 13-PG <--> ATP + 3-PG
R25 : 12 3-PG <--> 2-PG
R26 : 12 2-PG <--> PEP
R33 : 6 2-OXO + NH3 + NADPH --> GLU + NADP
R38 : -12 MAL + NAD <--> OAA + NADH
R41 : 6 PYR + ATP + CO2 --> OAA + ADP
R43 : -12 OAA + ATP <--> PEP + ADP + CO2
R44 : 6 OAA + GLU <--> ASP + 2-OXO
R45 : 6 ASP + ATP --> ASP-P + ADP
R46 : 6 ASP-P + NADPH --> ASP-SA + NADP
R48 : 12 MAL + NADP <--> PYR + CO2 + NADPH
R53 : 6 ASP-SA + PYR --> DHP
R54 : 6 DHP + NADPH <--> NADP + PDC
R55 : 6 PDC + NH3 + NADPH <--> DMP + NADP
R59 : 6 DMP --> LYS + CO2
R60 : 7 GLCex + ATP --> G6P + ADP

R62 : 6 LYS --> #

B) mode with highest L-lysine yield including malic enzyme (R48) and pts (R8) activity but no glucokinase (R60) activity. $Y_{Lys/S}$ =0.833 (C-mol L-lysine)/(C-mol glucose)

R# : Flux reaction

R1 : 6.0 # --> GLCex
R3 : 1.0 # --> O2
R4 : 10.0 # --> NH3
R7 : 6.0 CO2 --> #
R8 : 6.0 PEP + GLCex --> PYR + G6P
R11 : 6.0 G6P + NADP --> GLC-LAC + NADPH
R12 : 6.0 GLC-LAC --> 6-P-Gluconate
R13 : 6.0 6-P-Gluconate + NADP --> RIB-5P + CO2 + NADPH
R14 : 4.0 RIB-5P <--> XYL-5P
R15 : 2.0 RIB-5P <--> RIBO-5P
R16 : -2.0 S7P + GA3P <--> RIBO-5P + XYL-5P
R17 : 2.0 S7P + GA3P <--> E-4P + F6P
R18 : -2.0 F6P + GA3P <--> E-4P + XYL-5P
R19 : 4.0 ATP + F6P --> ADP + F-16-BP
R21 : 4.0 F-16-BP <--> GA3P + DAHP
R22 : 4.0 DAHP <--> GA3P
R23 : 10.0 GA3P + NAD <--> 13-PG + NADH
R24 : 10.0 ADP + 13-PG <--> ATP + 3-PG
R25 : 10.0 3-PG <--> 2-PG
R26 : 10.0 2-PG <--> PEP
R33 : 5.0 2-OXO + NH3 + NADPH --> GLU + NADP
R38 : -8.0 MAL + NAD <--> OAA + NADH
R41 : 9.0 PYR + ATP + CO2 --> OAA + ADP
R43 : -4.0 OAA + ATP <--> PEP + ADP + CO2
R44 : 5.0 OAA + GLU <--> ASP + 2-OXO
R45 : 5.0 ASP + ATP --> ASP-P + ADP
R46 : 5.0 ASP-P + NADPH --> ASP-SA + NADP
R48 : 8.0 MAL + NADP <--> PYR + CO2 + NADPH
R49 : 1.0 2 NADH + O2 + 4 ADP --> 2 NAD + 4 ATP
R53 : 5.0 ASP-SA + PYR --> DHP
R54 : 5.0 DHP + NADPH <--> NADP + PDC
R55 : 5.0 PDC + NH3 + NADPH <--> DMP + NADP
R59 : 5.0 DMP --> LYS + CO2
R62 : 5.0 LYS --> #

Table S 3.4: Examples of elementary flux modes with highest L-lysine yields during growth with simultaneous consumption of glucose and lactate (only fluxes with non-zero values are listed). A – mode with highest yield mode including malic enzyme activity, B – mode highest yield including malic enzyme activity and pts but without glucokinase, C – mode with highest yield excluding malic enzyme activity and using only pts.

A) mode with highest yield mode including malic enzyme activity (R48). $Y_{Lys/S}$ =0.848 (C-mol L-lysine)/(C-mol glucose)

R# : Flux reaction
R1 : 15.0 # --> GLCex
R2 : 3.0 # --> LACex
R3 : 1.0 # --> O2
R4 : 28.0 # --> NH3
R7 : 15.0 CO2 --> #
R9 : 3.0 LACex + NAD --> PYR + NADH
R11 : 15.0 G6P + NADP --> GLC-LAC + NADPH
R12 : 15.0 GLC-LAC --> 6-P-Gluconate
R13 : 15.0 6-P-Gluconate + NADP --> RIB-5P + CO2 + NADPH
R14 : 10.0 RIB-5P <--> XYL-5P
R16 : -5.0 S7P + GA3P <--> RIBO-5P + XYL-5P
R17 : 5.0 S7P + GA3P <--> E-4P + F6P
R18 : -5.0 F6P + GA3P <--> E-4P + XYL-5P
R19 : 10.0 ATP + F6P --> ADP + F-16-BP
R21 : 10.0 F-16-BP <--> GA3P + DAHP
R22 : 10.0 DAHP <--> GA3P
R23 : 25.0 GA3P + NAD <--> 13-PG + NADH
R24 : 25.0 ADP + 13-PG <--> ATP + 3-PG
R25 : 25.0 3-PG <--> 2-PG
R26 : 25.0 2-PG <--> PEP
R33 : 14.0 2-OXO + NH3 + NADPH --> GLU + NADP
R38 : -26.0 MAL + NAD <--> OAA + NADH
R41 : 15.0 PYR + ATP + CO2 --> OAA + ADP
R43 : -25.0 OAA + ATP <--> PEP + ADP + CO2
R44 : 14.0 OAA + GLU <--> ASP + 2-OXO
R45 : 14.0 ASP + ATP --> ASP-P + ADP
R46 : 14.0 ASP-P + NADPH --> ASP-SA + NADP
R48 : 26.0 MAL + NADP <--> PYR + CO2 + NADPH
R49 : 1.0 2 NADH + O2 + 4 ADP --> 2 NAD + 4 ATP
R53 : 14.0 ASP-SA + PYR --> DHP
R54 : 14.0 DHP + NADPH <--> NADP + PDC

R55 : 14.0 PDC + NH3 + NADPH <--> DMP + NADP
R59 : 14.0 DMP --> LYS + CO2
R60 : 15.0 GLCex + ATP --> G6P + ADP
R62 : 14.0 LYS --> #

B) mode highest yield including malic enzyme (R48) and pts (R8) activity but no glucokinase (R60) activity. $Y_{Lys/S}$ =0.792 (C-mol L-lysine)/(C-mol glucose+C-mol lactate)

R# : Flux reaction
R1 : 1.0 # --> GLCex
R2 : 2.8 # --> LACex
R3 : 1.1 # --> O2
R4 : 3.8 # --> NH3
R7 : 3.0 CO2 --> #
R8 : 1.0 PEP + GLCex --> PYR + G6P
R9 : 2.8 LACex + NAD --> PYR + NADH
R10 : -2.0 G6P <--> F6P
R11 : 3.0 G6P + NADP --> GLC-LAC + NADPH
R12 : 3.0 GLC-LAC --> 6-P-Gluconate
R13 : 3.0 6-P-Gluconate + NADP --> RIB-5P + CO2 + NADPH
R14 : 2.0 RIB-5P <--> XYL-5P
R15 : 1.0 RIB-5P <--> RIBO-5P
R16 : -1.0 S7P + GA3P <--> RIBO-5P + XYL-5P
R17 : 1.0 S7P + GA3P <--> E-4P + F6P
R18 : -1.0 F6P + GA3P <--> E-4P + XYL-5P
R23 : 1.0 GA3P + NAD <--> 13-PG + NADH
R24 : 1.0 ADP + 13-PG <--> ATP + 3-PG
R25 : 1.0 3-PG <--> 2-PG
R26 : 1.0 2-PG <--> PEP
R33 : 1.9 2-OXO + NH3 + NADPH --> GLU + NADP
R38 : -1.6 MAL + NAD <--> OAA + NADH
R41 : 3.5 PYR + ATP + CO2 --> OAA + ADP
R44 : 1.9 OAA + GLU <--> ASP + 2-OXO
R45 : 1.9 ASP + ATP --> ASP-P + ADP
R46 : 1.9 ASP-P + NADPH --> ASP-SA + NADP
R48 : 1.6 MAL + NADP <--> PYR + CO2 + NADPH
R49 : 1.1 2 NADH + O2 + 4 ADP --> 2 NAD + 4 ATP
R53 : 1.9 ASP-SA + PYR --> DHP
R54 : 1.9 DHP + NADPH <--> NADP + PDC
R55 : 1.9 PDC + NH3 + NADPH <--> DMP + NADP
R59 : 1.9 DMP --> LYS + CO2
R62 : 1.9 LYS --> #

C) Mode with highest yield excluding malic enzyme activity (R48) and without glucokinase (R60) activity. $Y_{Lys/S}$ =0.75 (C-mol L-lysine)/(C-mol glucose+C-mol lactate)

R# : Flux reaction

R1 : 1.0 # --> GLCex
R2 : 2.0 # --> LACex
R3 : 1.5 # --> O2
R4 : 3.0 # --> NH3
R7 : 3.0 CO2 --> #
R8 : 1.0 PEP + GLCex --> PYR + G6P
R9 : 2.0 LACex + NAD --> PYR + NADH
R10 : -2.0 G6P <--> F6P
R11 : 3.0 G6P + NADP --> GLC-LAC + NADPH
R12 : 3.0 GLC-LAC --> 6-P-Gluconate
R13 : 3.0 6-P-Gluconate + NADP --> RIB-5P + CO2 + NADPH
R14 : 2.0 RIB-5P <--> XYL-5P
R15 : 1.0 RIB-5P <--> RIBO-5P
R16 : -1.0 S7P + GA3P <--> RIBO-5P + XYL-5P
R17 : 1.0 S7P + GA3P <--> E-4P + F6P
R18 : -1.0 F6P + GA3P <--> E-4P + XYL-5P
R23 : 1.0 GA3P + NAD <--> 13-PG + NADH
R24 : 1.0 ADP + 13-PG <--> ATP + 3-PG
R25 : 1.0 3-PG <--> 2-PG
R26 : 1.0 2-PG <--> PEP
R27 : 4.0 PEP + ADP --> PYR + ATP
R33 : 1.5 2-OXO + NH3 + NADPH --> GLU + NADP
R41 : 5.5 PYR + ATP + CO2 --> OAA + ADP
R42 : 0.0 PEP + CO2 --> OAA
R43 : 4.0 OAA + ATP <--> PEP + ADP + CO2
R44 : 1.5 OAA + GLU <--> ASP + 2-OXO
R45 : 1.5 ASP + ATP --> ASP-P + ADP
R46 : 1.5 ASP-P + NADPH --> ASP-SA + NADP
R49 : 1.5 2 NADH + O2 + 4 ADP --> 2 NAD + 4 ATP
R53 : 1.5 ASP-SA + PYR --> DHP
R54 : 1.5 DHP + NADPH <--> NADP + PDC
R55 : 1.5 PDC + NH3 + NADPH <--> DMP + NADP
R59 : 1.5 DMP --> LYS + CO2
R61 : 1.5 LYS --> #

Supplementary Figures

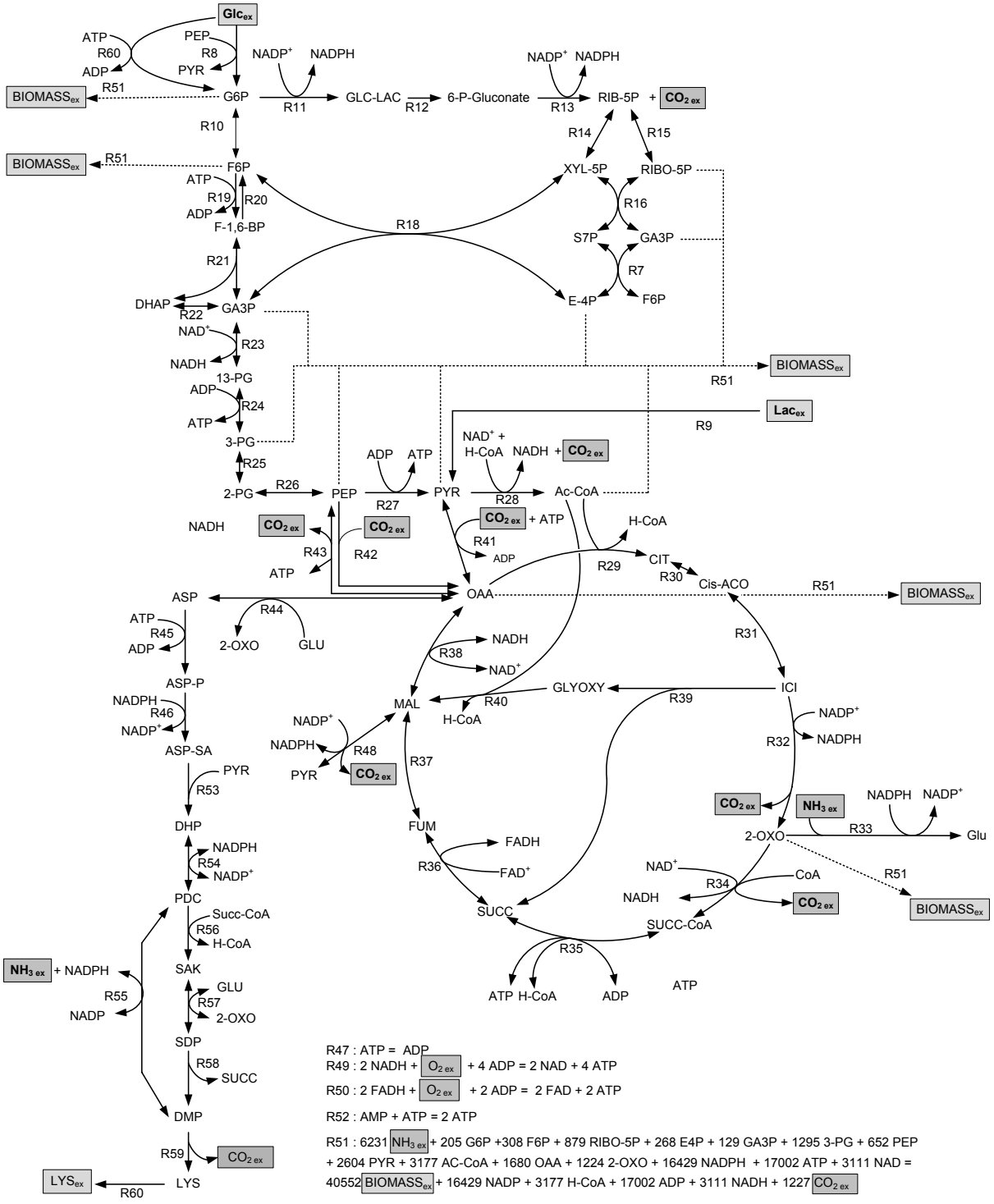

Figure S 3.1: Metabolic network for the growth and production of biomass using glucose and lactate as carbon sources (List of reactions is provided in supplementary Table S 3.1).

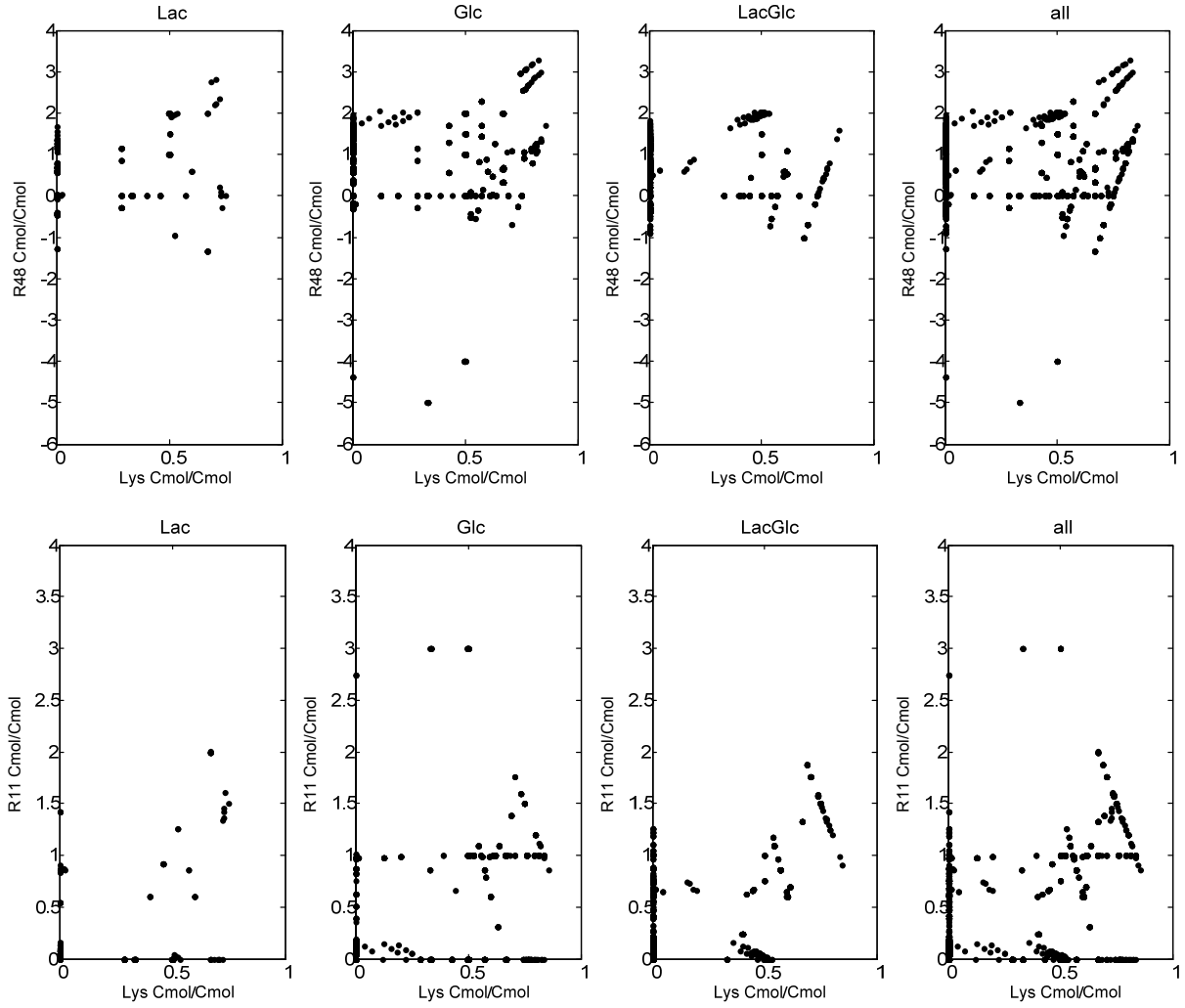

Figure S 3.2: Relative carbon flux through malic enzyme (R48) and glucose-6-phosphate dehydrogenase (R11) and function of relative carbon flux to L-lysine (R61). Fluxes are related to total carbon uptake rate. Lac –growth only on lactate, Glc – growth only on glucose, LacGlc – modes with simultaneous uptake of lactate and glucose, all – all possible substrate variations cumulated.

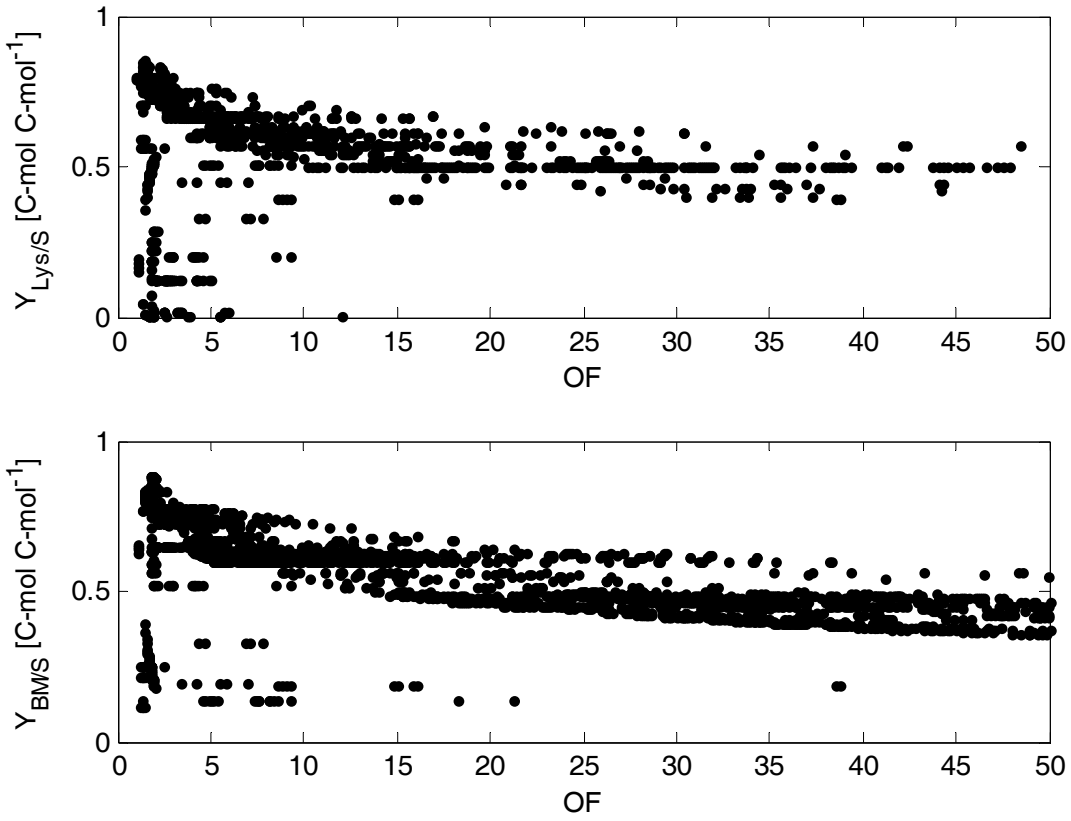

Figure S 3.3: Carbon yields of L-lysine, $Y_{Lys/S}$, and biomass, $Y_{BM/S}$, given in C-moles per C-mol as a function of the objective function, OF, specified in Equation 1.

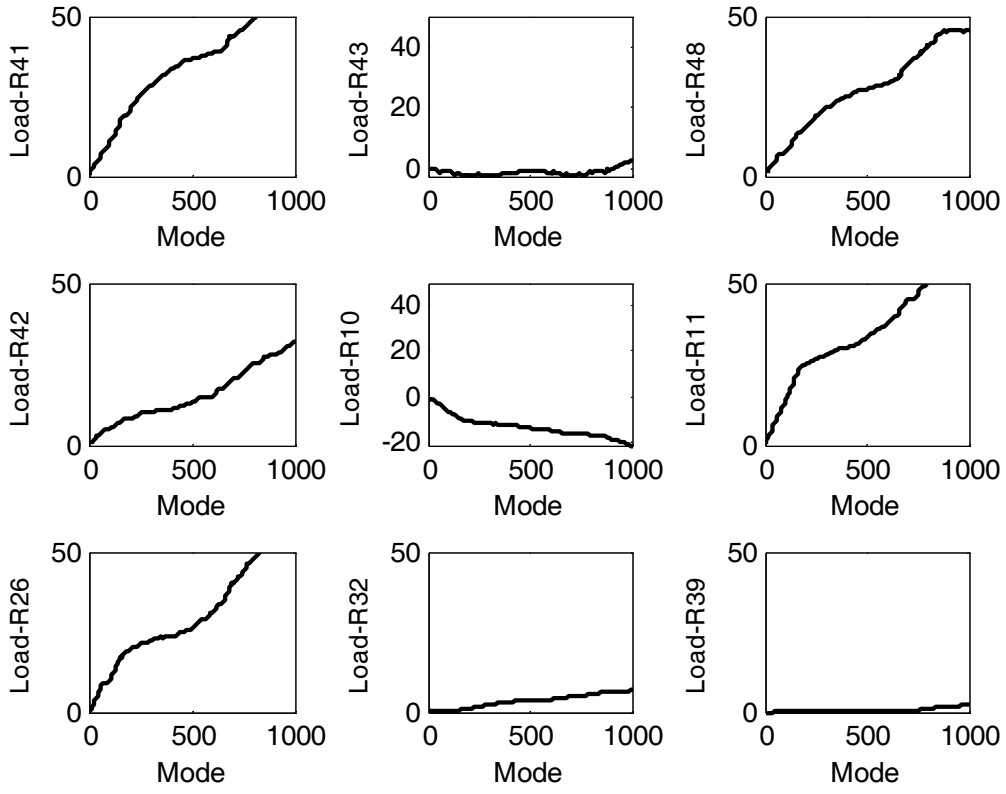

Figure S 3.4: Elementary modes ranked with increasing objective function, OF, and selected cumulative enzyme loadings as defined in Equation 2 for modes with growth on lactate, glucose or mixtures thereof. Reaction numbers are defined in supplementary Figure S 3.1 and supplementary Table S 3.1.

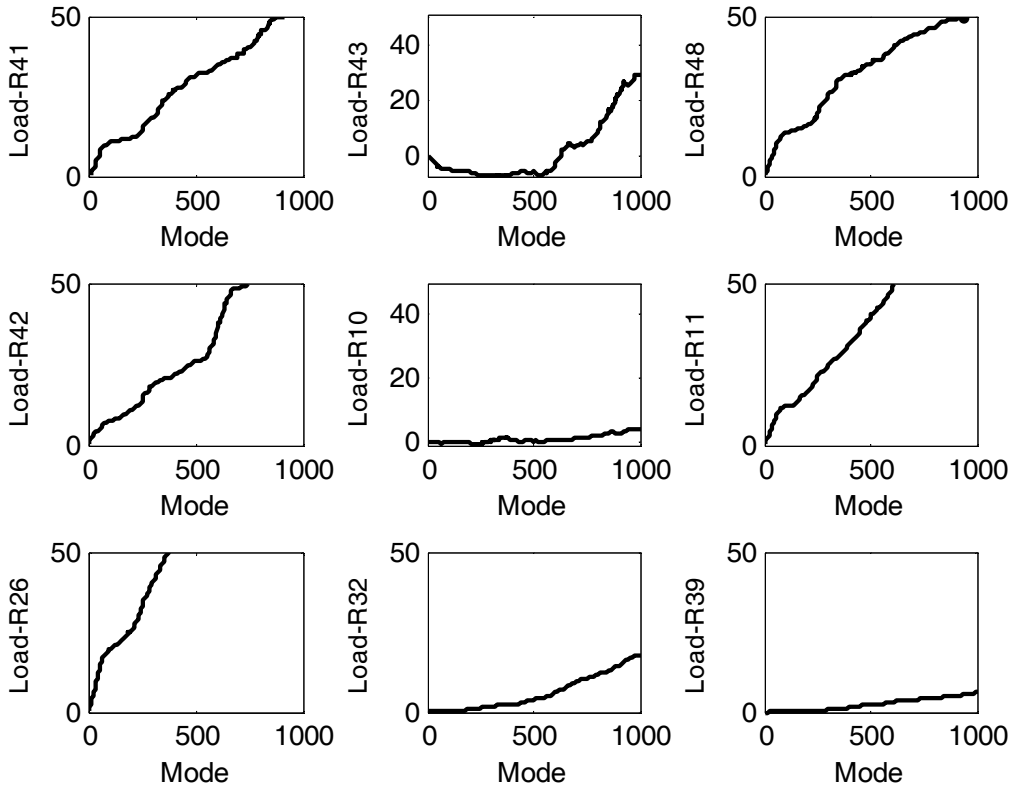

Figure S 3.5: Elementary modes ranked with increasing objective function, OF, and selected cumulative enzyme loadings as defined in Equation 2 for modes with only uptake of glucose. Reaction numbers are defined in Figure S 3.1 and supplementary Table S 3.1.

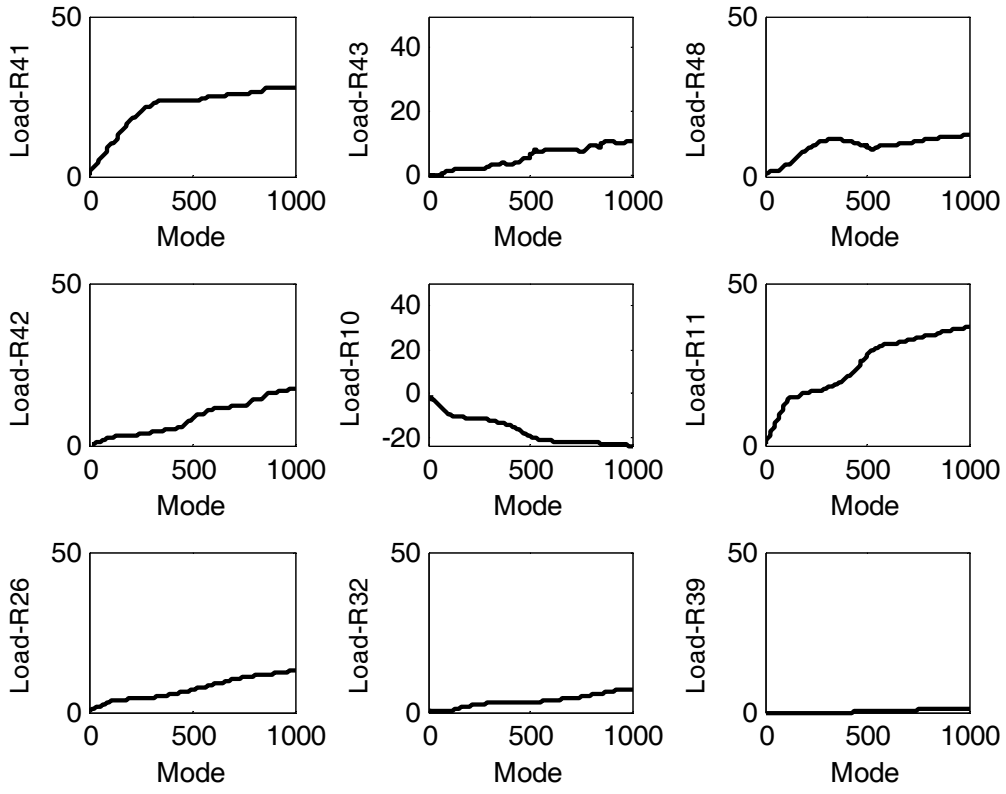

Figure S 3.6: Elementary modes ranked with increasing objective function, OF, and selected cumulative enzyme loadings as defined in Equation 2 for modes with simultaneous uptake of glucose and lactate. Reaction numbers are defined in Figure S 3.1 and supplementary Table S 3.1.

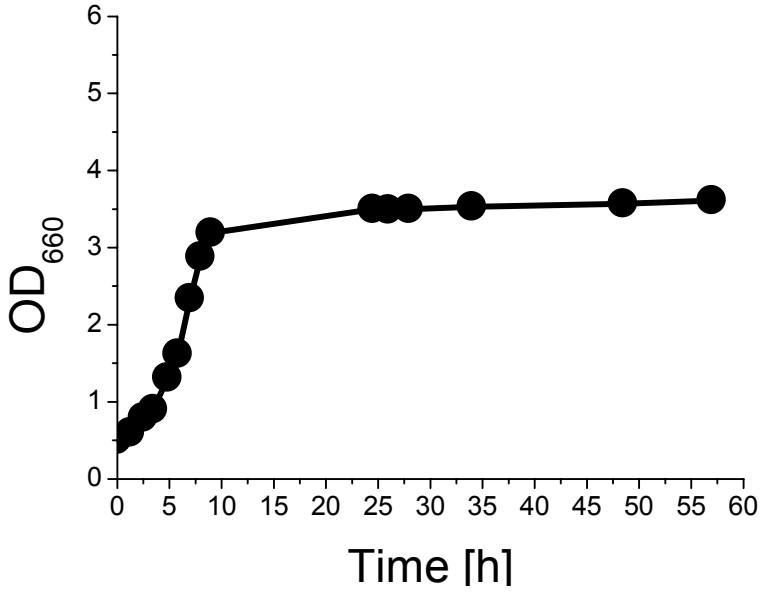

Figure S 3.7: Cultivation profile of *C. glutamicum* ATCC 13032 *lysC*^{fbr} on D,L-lactate

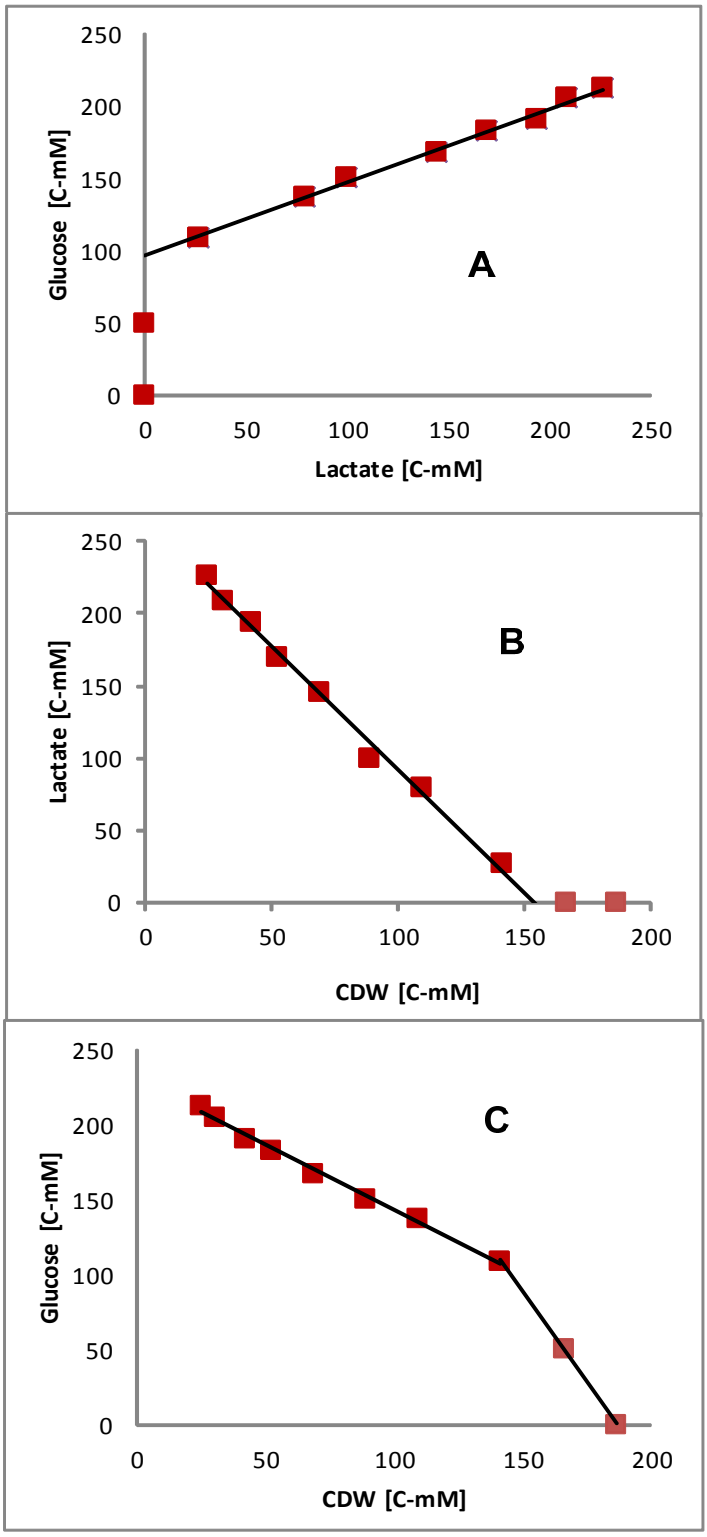

Figure S 3.8: Yield plots of growth and L-lysine production of *C. glutamicum* ATCC 13032 *lysC*^{fbr}*dld*$_{Psod}$*pyc*$_{Psod}$*malE*$_{Psod}$ on a mixture of D, L-lactate and glucose as depicted in Figure 3.5. A – glucose and lactate concentrations; B – cell dry weight, CDW and lactate; C – cell dry weight and glucose.

Supplementary material Chapter 4

Supplementary Figures

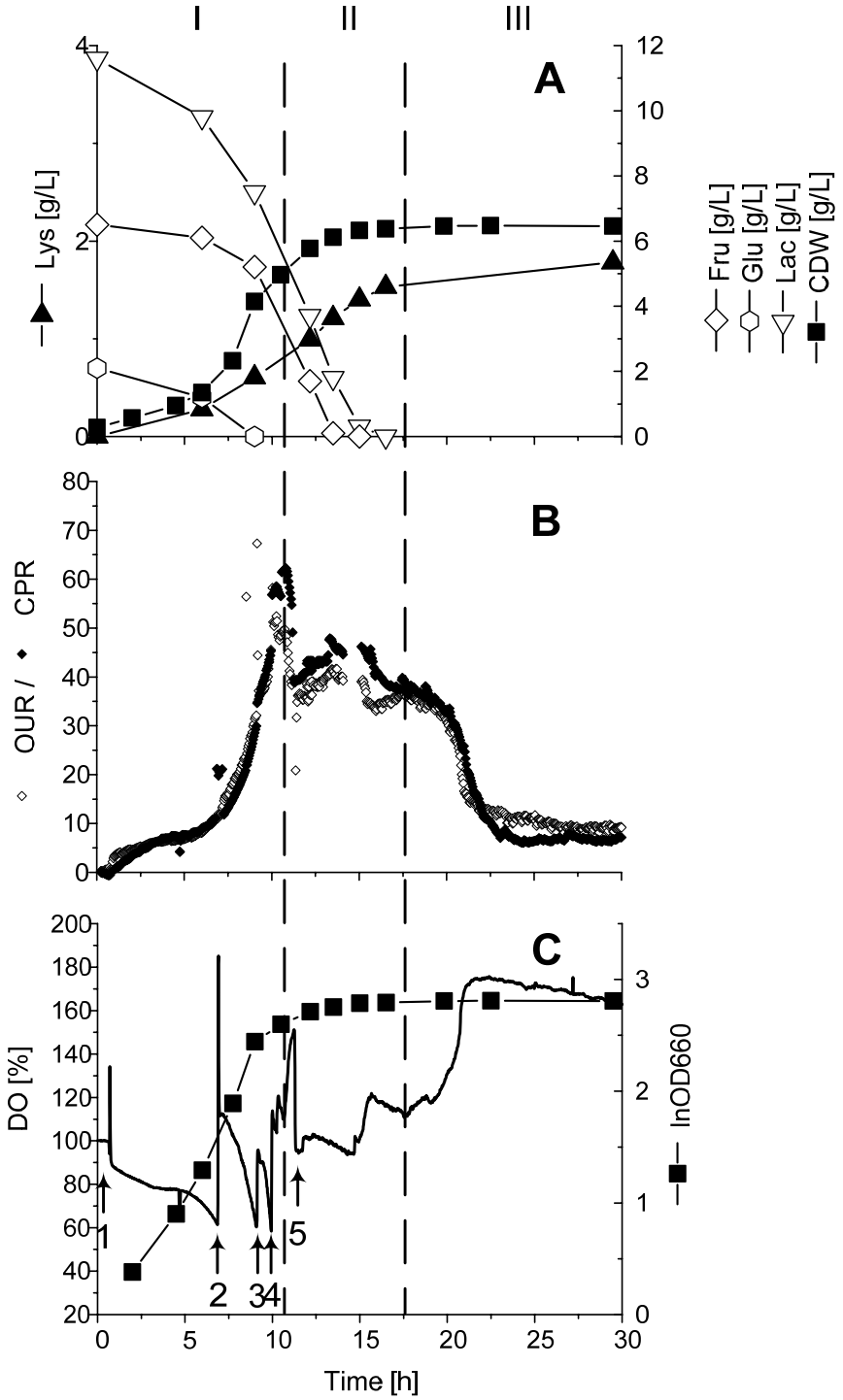

Figure S 4.1: Batch cultivation of L-lysine producing *C. glutamicum SL* on 1:4 diluted grass silage juice in the bioreactor using impeller 2 at a stirring speed of 600 rpm and a constant air flow rate of 124 ml min^{-1} (0.124 vvm) and additional oxygenation with

pure oxygen as in (C). (A) Concentrations of substrates fructose (Fru), glucose (Glu) and lactate (Lac), of product L-lysine (Lys) and of biomass provided as cell dry weight (CDW). (B) Oxygen uptake rate (OUR) and carbon dioxide production rate (CPR). (C) Optical density (OD) and dissolved oxygen concentration (DO – full line) given in % air saturation. Additional pure oxygen flow rate: 1 – 0 ml min^{-1}, 2 –16 ml min^{-1}, 3 –36 ml min^{-1}, 4 – 76 ml min^{-1} ml min^{-1}, 5 – 116 ml min^{-1}. Dashed lines indicate identified growth phases I, II and III.

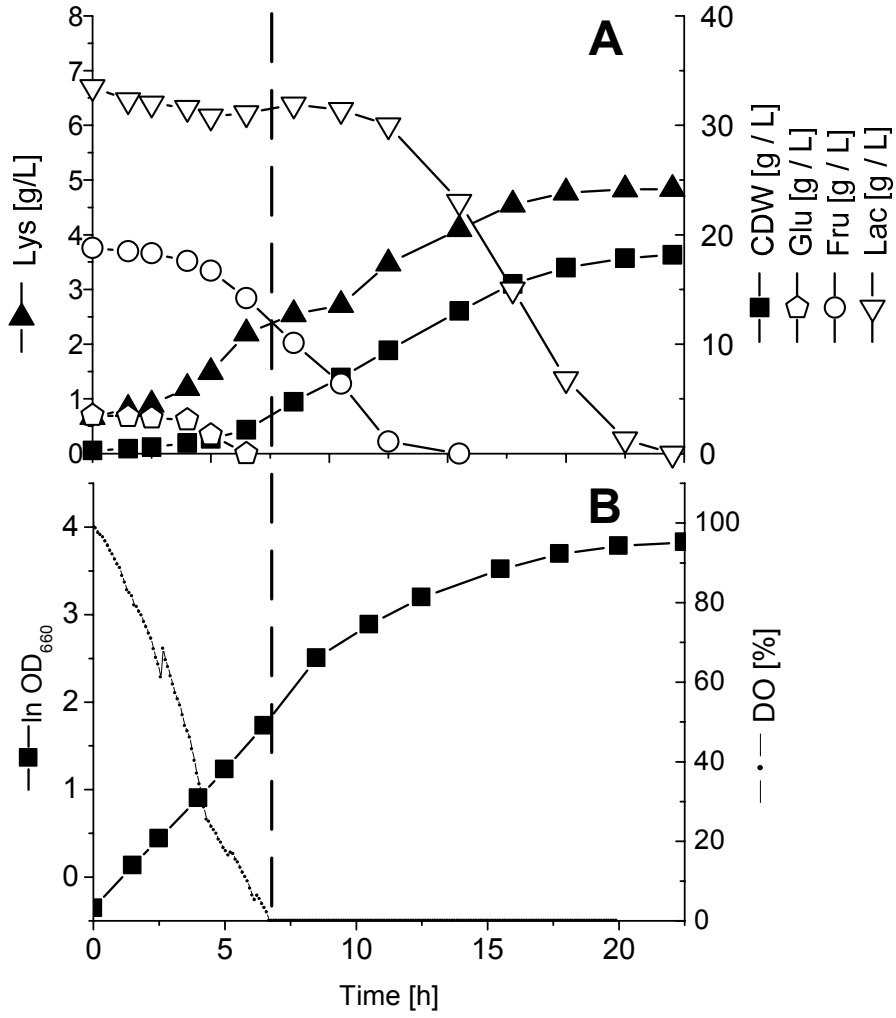

Figure S 4.2: Profile of shake flask cultivation of L-lysine producing *C. glutamicum SL* on grass silage juice. (A) Concentrations of substrates fructose (Fru), glucose (Glu) and lactate (Lac), of product L-lysine (Lys) and of biomass provided as cell dry weight (CDW). (B) Optical density (OD) and dissolved oxygen concentration (DO –

full line) given in % air saturation. Shaking rate: 230 rpm. Dashed lines indicate identified growth phases I and II.

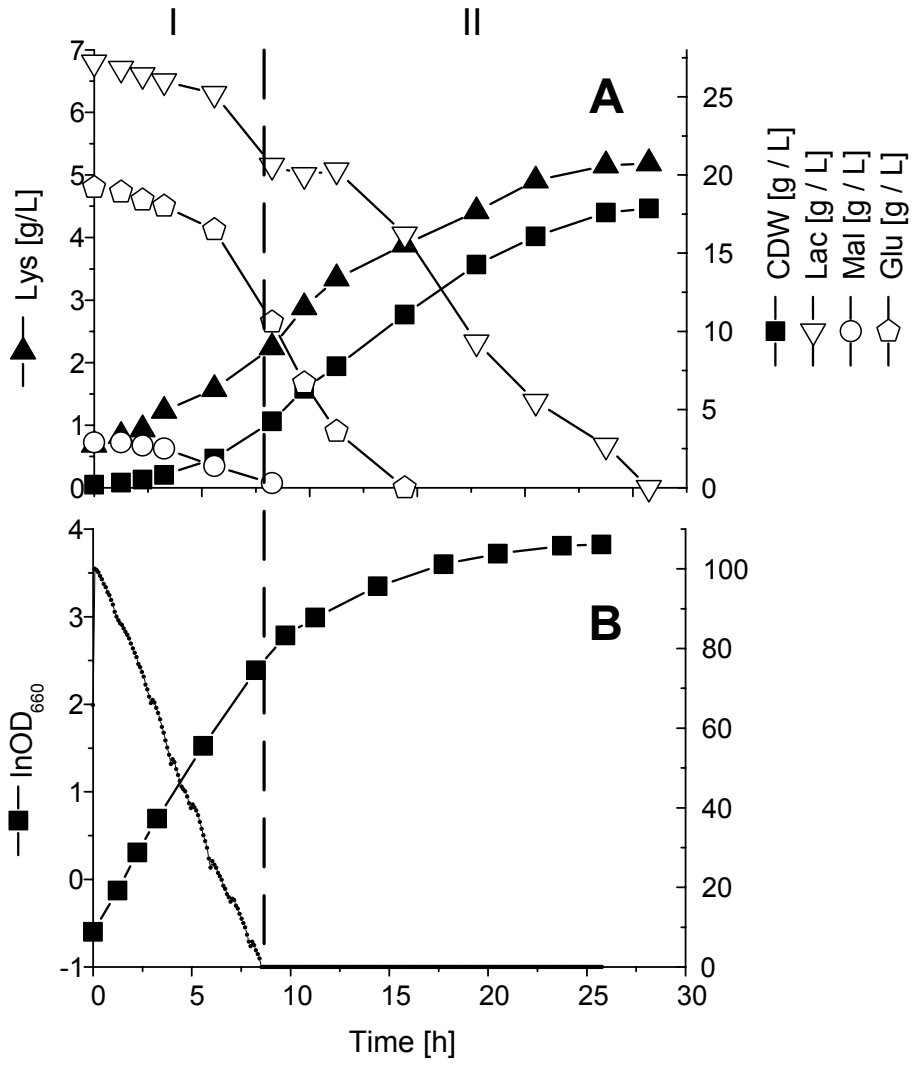

Figure S 4.3: Profile of shake flask cultivation of L-lysine producing *C. glutamicum SL* on corn silage juice. (A) Concentrations of substrates maltose (Mal), glucose (Glu) and lactate (Lac), of product L-lysine (Lys) and of biomass provided as cell dry weight (CDW). (B) Optical density (OD) and dissolved oxygen concentration (DO – full line) given in % air saturation. Shaking rate: 230 rpm. Dashed lines indicate identified growth phases I and II.

Curriculum vitae

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Aktuelle Tätigkeit

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Publikationen

Neuner, A., Wagner, I., Sieker, T., Ulber, R., Schneider, K., Peifer, S., Heinzle, E. (2012). Production of L-lysine on different silage juices using genetically engineered *Corynebacterium glutamicum*. *J Biotechnol* , In press.

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