INTRODUCTION

1 General Introduction

With a world market of 900,000 t/a, the essential amino acid L-lysine is one of the most important amino acids in biotechnology (Kohl and Tauch, 2009). It is mainly applied as supplement to animal feed (Anastassiadis, 2007), which is typically based on corn, wheat or barley and poor in lysine. The supplementation of such feed materials with a lysine rich source leads to optimized growth of e.g. pigs or chicken. The direct addition of lysine hereby has proven especially valuable (Kircher and Pfefferle, 2001). It does not cause an extra uptake and metabolization of other amino acids beyond their need so that superfluous formation of ammonia and environmental burden by increased nitrogen loads in the manure is avoided. The continuing development of an increased consumption of white meat in various countries of the western as well as the eastern world has led to an enormous market growth for lysine during the past decades. Since only the L-isomer of lysine is effective as feed supplement, all manufacturing processes from early on utilize fermentative production (Ikeda, 2003). Fermentation as technique for the industrial production of amino acids emerged with the discovery of the glutamate secreting bacterium *Corynebacterium glutamicum* (Kinoshita, et al., 1957) displayed below. Nowadays amino acids have the largest market volume among the fermentation products generating $ 5.4 billion in 2008. By 2013 this is expected to reach $ 7.8, corresponding to an annual growth rate of around 8 %.

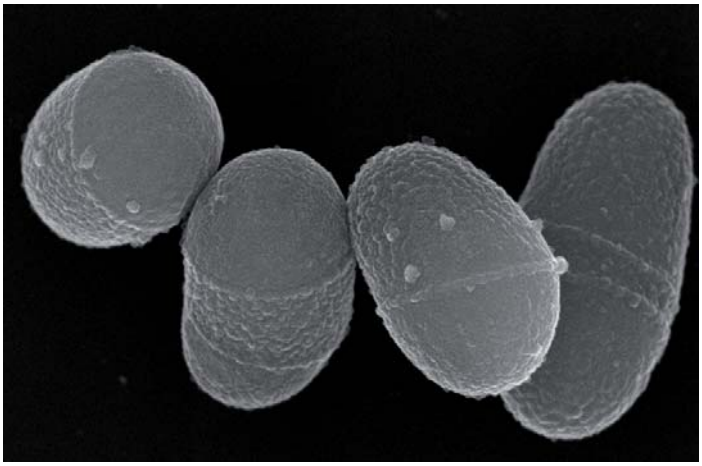

Figure 1: Raster electron micrograph of *Corynebacterium glutamicum* ATCC 13032 during exponential growth on minimal medium (Wittmann and Becker, 2007)

Within a few years from its discovery, the first lysine excreting mutants of *C. glutamicum* were available and applied for production in large scale (Kinoshita, et al., 1961). Until now, the lysine market is highly competitive, continuously forcing innovative developments to gain the leadership in this field. This stimulated an intensive research towards new technologies to establish high-efficient fermentation including optimization of the fermentation procedure, down-stream processing as well as strain engineering (Anastassiadis, 2007; Ikeda, 2003).

In the beginning the strains were classically engineered by an iterative approach of random mutagenesis with UV light or chemical mutagens and subsequent strain selection (Nakayama, et al., 1978). The key to success in these days was the use of toxic lysine analogues, such as S-(2-aminoethyl) cysteine, to select for feedback resistant strains (Nakayama and Araki, 1973). These classical strains typically shared point mutations in the aspartokinase gene, which release the encoded enzyme from feedback inhibition by lysine and threonine (Kalinowski, et al., 1991; Thierbach, et al., 1990). Remarkable production properties such as a conversion yield up to 50 % and a lysine·HCl titre of 100 g L^{-1} are achieved with such classically derived strains (Leuchtenberger, et al., 2005). This was, however, typically linked to extensive fermentation times of 2 – 3 days, limiting productivity. Additionally, auxotrophy and the weak stress tolerance, resulting from undesired mutations which accumulated during strain development (Ohnishi, et al., 2002), further display severe disadvantages of conventional production strains. In recent years, revolutionary findings in recombinant DNA technology and molecular biology initiated a new era of strain engineering – rational optimization by metabolic engineering (Ikeda, et al., 2006; Jäger, et al., 1992; Liebl, et al., 1989). Logically, many of these studies have focused on optimization of the flux through the lysine biosynthesis by directly modifying enzymes of this pathway. The release of aspartate kinase from feedback control is today regarded as one of the most important features of industrial production strains. Beside modifications concerning pathway regulation, the intracellular activity of rate determining enzymes of the biosynthetic pathway is a key point for strain engineering (Cremer, et al., 1991; Jetten, et al., 1995). Strategies for increasing enzyme activity within the cell involve over expression by the use of stronger promoters, mutating the promoter sequences or regulatory regions upstream of the gene, or increasing the copy number of the coding gene (Anastassiadis, 2007; Vasicova, et al., 1999). Plasmid-related over expression in this context is appropriate to achieve higher enzyme activities and better lysine yields (Eggeling, et al., 1998) but can hardly be applied in an industrial process.

Identification of beneficial targets apart from the biosynthetic pathway itself became soon necessary to abolish bottlenecks within the precursor and co-factor supply towards creation of

competitive production strains and also to circumvent the large patent coverage of the lysine biosynthetic pathway. This is, however, more challenging and requires understanding of the organism on a systems level. In this regard, the availability of the genome sequence of *Corynebacterium glutamicum* has been a mile stone for metabolic engineering (Haberhauer, et al., 2001; Kalinowski, et al., 2003; Ohnishi, et al., 2002; Pompejus, et al., 2002). It provided the basis for (i) genome breeding by comparative sequence analysis between classically derived production strains and the wild type (Ohnishi, et al., 2002), (ii) a detailed *in silico* reconstruction of the metabolic network of *C. glutamicum* (Kjeldsen and Nielsen, 2009) including stoichiometric modelling approaches to analyze the theoretical production capacity as well as metabolic pathways involved (Krömer, et al., 2006; Wittmann and Becker, 2007), and (iii) the discovery of transcriptional regulatory networks by means of specific sequence motives within the genome (Kohl and Tauch, 2009). These models, however, are not applicable to predict the activity of the metabolic pathways *in vivo*, i.e. the fluxome, as key characteristic for systems understanding and guidance of strain engineering. Flux analysis is a central element of metabolic engineering (Stephanopoulos, 1999), as indicated by the impressive progress to estimate metabolic fluxes *in viv*o (Christensen and Nielsen, 2000; Christensen, et al., 2000; Frick and Wittmann, 2005; Van Dien, et al., 2006; Wittmann, 2007; Wittmann and Heinzle, 2002). Beyond the fascinating insight into the biological system, ^{13}C metabolic flux analysis has proven especially useful for strain characterization and identification of beneficial targets for lysine production (Kiefer, et al., 2004; Wittmann and Heinzle, 2002). Together with complementary findings from determination of the active set of genes (transcriptome) (Glanemann, et al., 2003; Hayashi, et al., 2006) and proteins (proteome) (Bendt, et al., 2003; Schaffer, et al., 2001) and from quantification of intracellular metabolite levels (metabolome) (Borner, et al., 2007) an extensive data set is provided to gain a deep insight into cell physiology on a global level. This systems-oriented approach displays an excellent platform for metabolic engineering (Lee, et al., 2005).

2 Objectives

The aim of the present work was the optimization of lysine production by rational strain engineering of the production microorganism *Corynebacterium glutamicum* using a systems-oriented approach. As the raw material costs account for the major production costs in industrial lysine production, strain optimization, thus aimed at an increasing lysine yield, titre and productivity. The central strategy aimed at state-of-art technologies to unravel the metabolic and regulatory state of *C. glutamicum* on a systems oriented level and use the

obtained knowledge to identify genetic targets towards optimal production performance. In *C. glutamicum* lysine biosynthesis is closely connected to the central metabolism via the requirement of the carbon precursors and NADPH as reducing power. Due to this, the study included the reactions of the central metabolic pathways as promising targets for strain optimization. The strategies focussed on the NADPH metabolism, TCA cycle, engineering of oxaloacetate supply as well as lysine biosynthesis. First, the value of several engineering strategies towards improved lysine production should be investigated by target evaluation in lysine producing strains. The modified strains should be investigated in detail by comparative cultivation experiments as well as on the level of transcriptome, metabolome, and fluxome to (i) gain a deep insight into the cellular physiology, (ii) to estimate the benefit of the applied strategy with regard to industrial application and (iii) to provide valuable information for the rational design of a production strain. Finally, the work aimed at the creation of a lysine hyper-producing strain based on the wild type of *C. glutamicum*. This should be obtained by combination of an exclusive set of beneficial modifications identified in this work, in a single strain. Ideally, the tailor-made cell factory should have a high carbon conversion yield as well as a high space-time yield, a high final lysine titre, good growth behaviour and lack of any by-product formation. These production characteristics would ensure a fast and efficient conversion of the supplied substrates and thus a cost-effective production of lysine. Beyond lysine production, the present work should also demonstrate the high potential of the novel concept of systems metabolic engineering, combining state-of-art omics tools for strain characterization with targeted genetic engineering to obtain superior strains for bio-based production of chemicals.