ulm university universität
uulm

Analysis of the biotechnological production of 2-ketomethylvalerate with *Corynebacterium glutamicum*

Dissertation zur Erlangung des Doktorgrades Dr. rer. nat. der Fakultät für Naturwissenschaften der Universität Ulm

vorgelegt von Christian Thomas Hagmann

aus Ulm

2011

Die vorliegende Arbeit wurde am Institut für Mikrobiologie und Biotechnologie der Universität Ulm unter Anleitung von Herrn Prof. Dr. Bernhard J. Eikmanns angefertigt.

Amtierender Dekan: Prof. Dr. Axel Groß

1. Gutachter: Prof. Dr. Bernhard J. Eikmanns
2. Gutachter: Prof. Dr. Peter Dürre

Tag der Promotion: 08.06.2011

Table of contents

TABLE OF CONTENTS **3**

ABSTRACT ..... **- 1 -**

1. INTRODUCTION ..... **- 3 -**

2. MATERIAL AND METHODS ..... **- 11 -**

2.1 Strains, plasmids and oligonucleotides ..... **- 11 -**

2.2 Chemicals and equipment ..... **- 14 -**

2.3 Nutrient solutions and culture conditions ..... **- 18 -**

2.4 Isolation of nucleic acids ..... **- 20 -**

2.5 Clean up of nucleic acid solutions ..... **- 23 -**

2.6 Transformation of *C. glutamicum* and *E. coli* cells ..... **- 24 -**

2.7 Enzymatic treatment of nucleic acids ..... **- 25 -**

2.8 Agarose gel electrophoresis ..... **- 27 -**

2.9 Determination of glucose concentration ..... **- 28 -**

2.10 Measurement of enzyme activities ..... **- 29 -**

2.11 Chromatographic separation ..... **- 34 -**

3. RESULTS ..... **- 38 -**

3.1 Influence of KMV on *C. glutamicum* ..... **- 38 -**

3.1.1 Effect of KMV and other branched-chain α-keto acids on the growth of *C. glutamicum* ..- 38 -

3.2 Degradation of branched-chain α-keto acids ..... **- 41 -**

3.2.1 Degradation of branched-chain α-keto acids by *C. glutamicum* during growth ..... - 41 -

3.2.2 Stability of branched-chain α-keto acids in the presence of corn steep liquor ..... - 44 -

3.2.3 Enzymatic degradation of branched-chain α-keto acids ..... - 47 -

3.2.4 Incubation of the branched-chain α-keto acids in “spent” medium ..... - 52 -

3.3 Characterization of key enzymes of the KMV biosynthesis ..... **- 54 -**

3.3.1 Characterization of the acetohydroxy acid synthase ..... - 54 -

3.3.2 Inhibition assays with the biosynthetic threonine dehydratase and branched-chain α-keto acids ..... - 57 -

3.4 KMV production with *C. glutamicum* ..... **- 60 -**

3.4.1 KMV production with 2-oxobutyrate as a co-substrate ..... - 60 -

3.4.2 Construction and analysis of a KMV production strain ..... - 63 -

3.4.3 KMV production under limitation of branched-chain amino acids ..... - 69 -

3.4.4 Fed-batch fermentation with glucose and 2-oxobutyrate ..... - 74 -

3.4.5 Fermentation of the KMV production strain with homoserine as second substrate - 76 -

3.4.6 Use of a feedback-resistant variant of the TD in the KMV production strain ..... - 77 -
3.4.7 Use of the catabolic TD (*tdcB*) of *E. coli* ..... - 80 -
3.4.8 Flux reduction into L-lysine biosynthesis ..... - 82 -
4. DISCUSSION ..... - 84 -
LITERATURE ..... - 93 -
ABBREVIATIONS ..... - 103 -
ZUSAMMENFASSUNG ..... - 106 -
SIDE PROJECT AND PUBLICATION ..... - 108 -
DANKSAGUNG ..... - 109 -
ERKLÄRUNG ..... - 110 -

Abstract

The current work deals with the biotechnological synthesis of α-ketomethylvalerate (KMV) with *Corynebacterium glutamicum*. KMV together with the two other branched-chain α-keto acids α-ketoisovalerate (KIV) and α-ketoisocaproate (KIC) is used as a pharmaceutical agent and also as ingredient of functional food. So far KMV has only been produced by chemical synthesis and the aim of this work was to initiate the development of a fermentative KMV production.

First the influence of KMV, KIV and KIC on the metabolism and the growth of *C. glutamicum* was investigated. When *C. glutamicum* was cultured in the presence of the branched-chain α-keto acids the growth rate and the final cell density were significantly lower than without these acids. Especially KMV had a strong impact on the growth as 50 mM led to an almost complete inhibition. The inhibition could be reverted by the supplementation of L-isoleucine, L-valine and L-leucine, and also by the other two branched-chain α-keto acids, indicating that a high concentration of KMV inhibits the biosynthesis of the branched-chain amino acids. The experiments also revealed that the branched-chain α-keto acids are degraded by *C. glutamicum* during growth. *C. glutamicum* WT quite efficiently converts them to the corresponding amino acids by transamination, but also in cultures of the transaminase B-negative strain *C. glutamicum* Δ*ilvE* a significant decrease of KMV, KIV, and KIC concentration was observed. Specific branched-chain α-keto acid converting enzymes could not be identified in *C. glutamicum*. The most plausible explanation would be an unspecific degradation catalyzed by other α-keto acid-converting enzymes.

Analysis of key enzymes of the KMV biosynthesis revealed that the pyruvate condensing reaction of the acetohydroxy acid synthase (AHAS) is competitively inhibited by 100 mM KMV. Positive for KMV production, the condensation reaction of 2-oxobutyrate plus pyruvate of the enzyme proved to be resistant against KMV. The condensation reaction of 2-oxobutyrate plus pyruvate of the AHAS was inhibited by branched-chain amino acids in the same way as the pyruvate condensing reaction. Remarkable was the decrease of the K_m-value for 2-oxobutyrate from about 6 mM to less than 2.5 mM in the presence of L-valine. Such a regulatory effect may secure the supply with L-isoleucine in the presence of high concentrations of L-valine. The threonine dehydratase which is known to be allosterically inhibited by L-isoleucine was also slightly inhibited by KMV, but presumably in a competitive manner.

KMV was produced with *C. glutamicum* Δ*ilvE* from glucose and 2-oxobutyrate as substrates with an efficient conversion of more than 70 % of the 2-oxobutyrate to KMV. The experiment

demonstrates that the synthesis pathway from 2-oxobutyrate towards KMV is open for KMV production. As a basis for the construction of a KMV production strain the L-lysine producer *C. glutamicum* DM1729 was employed. *C. glutamicum* DM1729 is deregulated in its pyruvate carboxylase and aspartate kinase activities leading to the formation of aspartate semialdehyde, a common intermediate of L-lysine and KMV biosynthesis. In this strain the *ilvE*-gene, encoding the transaminase B, was deleted, which lead to an about 65 % increased excretion of L-lysine. To redirect the carbon flow towards KMV synthesis the genes *hom*^{FBR}, *thrB*, *thrC* and *ilvA*, encoding a feedback-resistant version of the homoserine dehydrogenase, the homoserine kinase, the threonine synthase and the threonine dehydratase, were overexpressed on plasmids. The resulting strain *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) is the first described strain for the intentional production of KMV from glucose. At first the produced amounts of KMV were quite low, but by optimizing the medium composition and growth conditions a higher concentration could be achieved. Under limitation of branched-chain amino acids a maximal concentration of 35 mM KMV was produced from 4 % glucose as only carbon source.

1. Introduction

The Gram-positive *Corynebacterium glutamicum* was firstly isolated in the course of a study having the aim to find glutamate excreting microorganisms (Kinoshita *et al.*, 1957). The genus *Corynebacterium* derived its name from the typical morphology of *C. glutamicum*, forming an irregular short rod (*coryne*, greec for cudgel). *C. glutamicum* is a facultatively anaerobic, non-spore-forming, biotin-auxotrophic, immobile soil bacterium which needs the vitamin biotin for growth (Abe *et al.*, 1967; Takeno *et al.*, 2007). Phylogenetically it belongs to the family *Corynebacteriaceae* in the suborder *Corynebacterineae* that also includes the *Mycobacteriaceae* (Stackebrandt *et al.*, 1997; Liebl, 2005). Typical features of this suborder are a relatively high G+C content of the genome and a mycolic acid layer in the cell wall, which serves as an additional permeability barrier similar to the outer membrane of Gram-negative bacteria (Minnikin *et al.*, 1978; Bayan *et al.*, 2003; Daffé, 2005). In contrast to the closely related *C. diphtheriae*, *Mycobacterium tuberculosis* and *M. leprae*, *C. glutamicum* is a non-pathogenic bacterium, therefore being an appropriate model organism for the investigation of those important pathogenic bacteria (Funke *et al.*, 1997). Since 2003 the whole genome of *C. glutamicum* is sequenced, comprising of 3.28 mbp and possessing a G+C content of 53.8 % (Ikeda and Nakagawa, 2003; Kalinowski *et al.*, 2003).

C. glutamicum was discovered for its ability to excrete the amino acid glutamate, which is used as a flavor enhancer in food industry since the beginning of the 20th century. Under certain conditions such as biotin deprivation, addition of detergents or small amounts of penicillin large amounts of L-glutamate are excreted into the culture medium (Shiio *et al.*, 1962; Nunheimer et *al.*, 1970). Shortly after its discovery mutants, generated via UV-mutagenesis, were isolated, which produce L-lysine, an important feed stuff additive in animal nutrition (Kinoshita *et al.*, 1958). Since then *C. glutamicum* has gained rising industrial importance for the biotechnological production of L-glutamate and L-lysine (Leuchtenberger *et al.*, 2005). In 2009 about 2.16 mio tons of L-glutamate and 1.33 mio tons of L-lysine were produced in this way (Ajinomoto Co., Inc. estimate). Due to the large economic importance many scientific projects have the aim to enhance the productivity and to enlarge the product spectrum on other metabolites, among them the amino acids L-valine, L-leucine and L-isoleucine. Until recently the production strains have been attained by undirected mutagenesis. For further improvement of strains and to make new products accessible, directed mutagenesis became the method of choice. Several methods for the genetic modification of *C. glutamicum* (Kirchner and Tauch, 2003; Vertès *et al.*, 2005) and of course the sequencing of the genome allowed the deletion of genes or the enhancement of

their expression and also the expression of heterologous genes in *C. glutamicum*. Such intrusions require a large understanding for the metabolic pathways and their regulation, because only if metabolism, enzymes and their regulation are truly understood it is possible to redirect the carbon flux by specific mutations.

Central metabolism of *C. glutamicum*

The central metabolism of an organism is of special importance, because it enables it to form biomass, provides precursors and also the energy necessary for product formation in form of ATP and reduction equivalents. *C. glutamicum* is able to grow with various carbon sources such as sugars, alcohols and organic acids (Liebl, 1991). Since costs of substrates are of great importance, for industrial fermentations *C. glutamicum* is mostly grown with sugar-containing complex substrates such as cane molasses, beet molasses, or hydrolysates from corn, wheat or cassava, the type of sugar being dependent on the geographical location of the production plant (Hermann, 2003; Ikeda, 2003; Kimura, 2005; Kelle *et al.*, 2005). While molasses are more common in Europe, South America and China, starch hydrolysate from corn (corn syrup) is the most important carbon source in North America (Kimura, 2005). The uptake of glucose and also of fructose and sucrose is accomplished by phosphoenol pyruvate (PEP)-dependent sugar specific phosphotransferase systems (PTSs). The metabolic utilization of sugars is performed via the glycolysis, where glucose-6-phosphate is converted to pyruvate (figure 1). Part of the glucose-6-phosphate is oxidized in the pentose-phosphate pathway (PPP; Kiefer et al., 2004), where several different sugar-phosphates are formed that may be used in the anabolic metabolism. Remaining sugar-phosphates are channeled back into the glycolysis on the stage of fructose-6-phosphate and glycerol aldehyde-3-phosphate (GAP) (figure 1). The division of the flux in these two pathways can have a large influence on the biotechnological production of amino acids, because of the differences with the formed reduction equivalents. While NAD^+ is reduced to NADH + H^+ in the glycolysis, in the PPP NADPH + H^+ is formed, whose supply is an important factor for the synthesis of for instance L-lysine and L-isoleucine. Pyruvate is decarboxylated by the pyruvate dehydrogenase complex (PDHC) to the central metabolite acetyl-CoA, which is transferred into the tricarboxylic acid cycle (TCA cycle). The citrate synthase condenses acetyl-CoA and oxaloacetate to form citrate (figure 1). In the TCA cycle acetyl-CoA is oxidized to CO_2, thereby generating reduction equivalents for respiration and ATP formation. Next to this catabolic function, the TCA cycle is also of importance as source of precursors for the amino acid synthesis. 2-oxoglutarate and oxaloacetate are the precursors of the glutamate family and the aspartate family of amino acids, respectively. Since also pyruvate serves as precursor for amino acid synthesis, the pyruvate – acetyl-CoA – oxaloacetate node is of

special interest. This node comprises several enzyme catalyzed reactions next to the PDHC reaction. To compensate the drain off of oxaloacetate and 2-oxoglutarate for amino acid synthesis, the anaplerotic enzymes pyruvate carboxylase and PEP carboxylase are present, which synthesize oxaloacetate from pyruvate and PEP, respectively, whereupon HCO_3^- is fixed (Peters-Wendisch *et al.*, 1997 and 1993). The reverse reactions catalyzed by oxaloacetate decarboxylase and the PEP carboxykinase are part of the gluconeogenesis, which is active when *C. glutamicum* grows on acetate or other organic acids (Klaffl and Eikmanns, 2010; Riedel *et al.*, 2001).

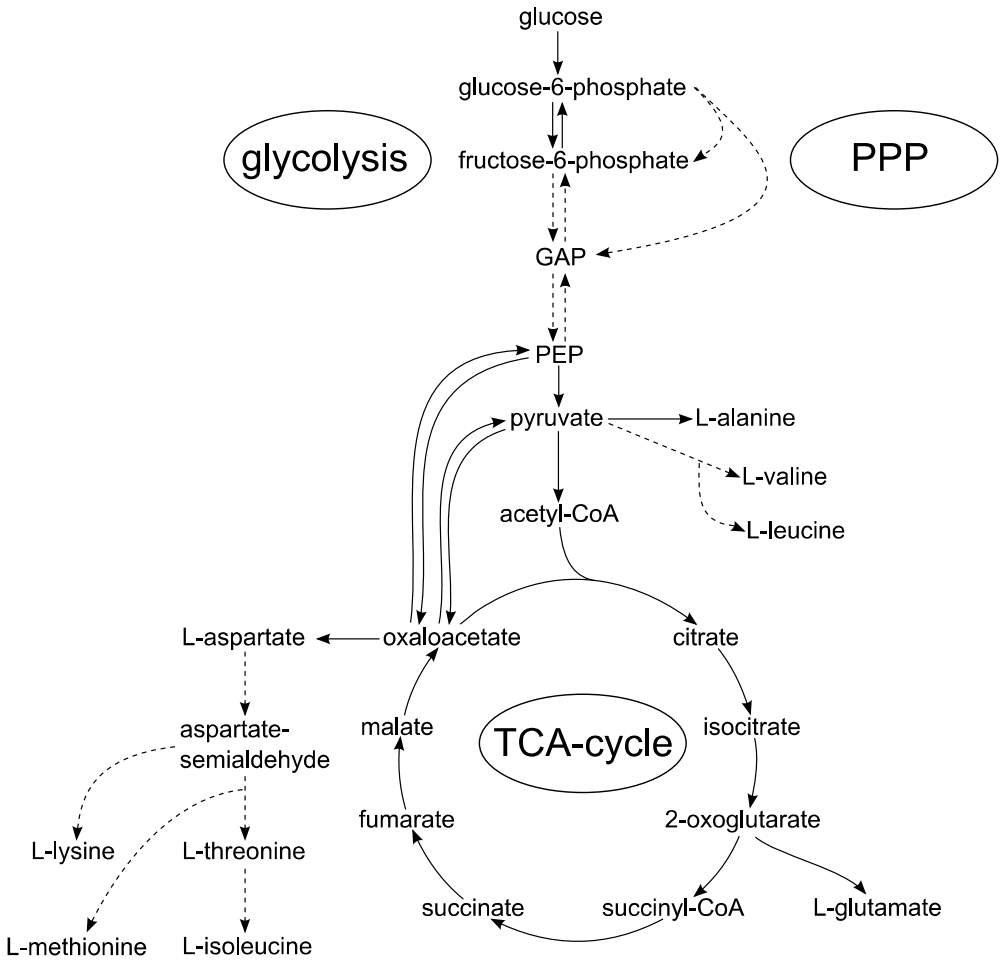

Figure 1: Central metabolism of *C. glutamicum*: Glycolysis (with gluconeogenesis), pentosephosphate pathway (PPP) and tricarboxylic acid cycle (TCA-cycle). Also shown are the amino acids of the pyruvate and the aspartate family. Straight arrows are single reactions, dashed arrows represent a set of several reactions. GAP, glycerol aldehyde-3-phosphate; PEP, phosphoenol pyruvate

Synthesis of α-ketomethylvalerate

α-ketomethylvalerate (KMV) is the precursor of L-isoleucine, an amino acid belonging to the aspartate family of amino acids. Also part of this family are L-aspartate, L-lysine, L-threonine, L-methionine and the non-proteinogenic L-homoserine and meso-diaminopimelate. The synthesis pathways for those metabolites form a network with several branching points (figure 2). The initial reaction into this metabolic pathway is the amination of oxaloacetate to form aspartate, catalyzed by the aspartate aminotransferase (Marienhagen *et al.*, 2005). The first reactions of the L-isoleucine biosynthesis, catalyzed by the aspartate kinase and the aspartate semialdehyde dehydrogenase, transform aspartate to aspartate semialdehyde. At this point the metabolic flux divides itself into the L-lysine and the L-threonine/L-isoleucine branches. The dihydrodipicolinat synthase, that condensates aspartate semialdehyde with pyruvate, leads to diaminopimelate, which is necessary for cell wall construction, and to lysine. On the other hand the homoserine dehydrogenase (HOM), the homoserine kinase and the threonine synthase catalyze the reactions for the synthesis of L-threonine. The product of the HOM, L-homoserine, is also the precursor for the synthesis of L-methionine. The proteinogenic amino acid L-threonine is an important intermediate of the L-isoleucine synthesis, since it is the substrate of the threonine dehydratase (TD). This enzyme, which desaminates L-threonine to 2-oxobutyrate, is the only enzyme which is unique for the L-isoleucine synthesis and not part of any other pathway. All following enzymes also catalyze reactions that are part of the L-valine and L-leucine synthesis. Those amino acids belong to the pyruvate family, but due to the common employment of enzymes they may be regarded as associated to the aspartate family of amino acids. The synthesis of KMV from 2-oxobutyrate and pyruvate is catalyzed by the three enzymes acetohydroxy acid synthase (AHAS, encoded by *ilvBN*), α-hydroxy acid isomeroreductase (AHAIR, encoded by *ilvC*) and dihydroxy acid dehydratase (DHAD, encoded by *ilvD*). The analog reactions of the L-valine synthesis lead to the formation of α-ketoisovalerate (KIV), the precursor of L-valine. KMV and KIV are transaminated with glutamate by the transaminase B (encoded by *ilvE*) to form L-isoleucine and L-valine. KIV can also be converted into α-ketoisocaproate (KIC) in three further reactions. KIC, another branched-chain α-keto acid, is the precursor of L-leucine and is also aminated by the transaminase B. As shown in figure 2, 2 ATP and 3 NADPH are required for the synthesis of one molecule L-isoleucine from 2-oxobutyrate and pyruvate. Additionally two more NADPH are needed for the synthesis of glutamate which is used as amino donor for the two transamination reactions.

Because of the many different end products and also because some amino acids such as L-threonine are the precursors of others, a complex regulation of gene expression and enzyme activity is necessary. The parallel catalysis of different reactions, part of the L-valine and L-

leucine synthesis, by the same enzymes makes the regulation even more complex. The metabolic network and its regulation have first been described in *Escherichia coli* (Wormser and Pardee, 1957; Freundlich *et al.*, 1962). Also for *C. glutamicum* a regulatory network for the aspartate

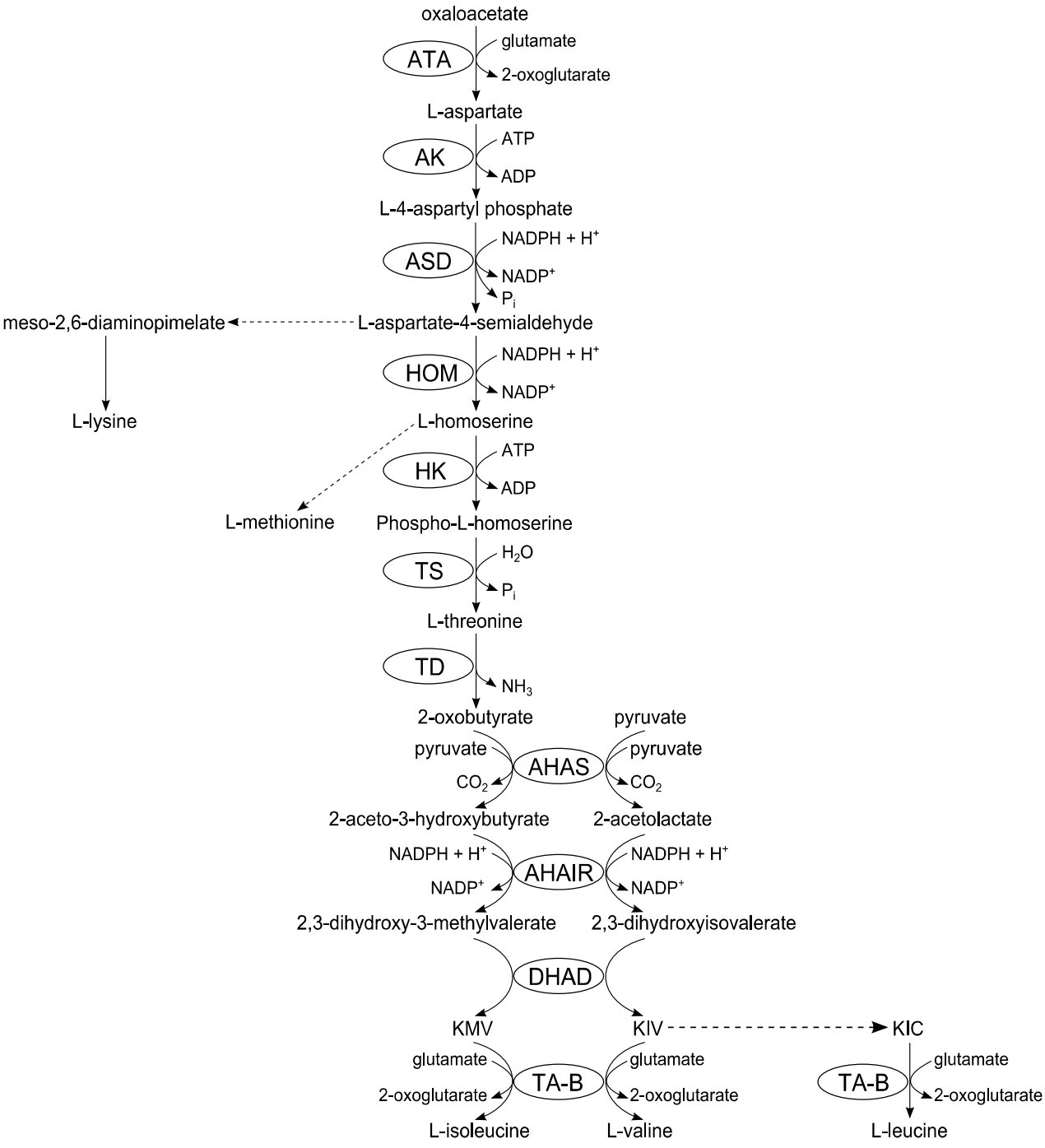

Figure 2: Synthesis pathway of amino acids of the aspartate family. ATA, aspartate amino transferase; AK, aspartate kinase; ASD, aspartate semialdehyde dehydrogenase; HOM, homoserine dehydrogenase; HK, homoserine kinase; TS, threonine synthase; TD, threonine dehydratase; AHAS, acetohydroxy acid synthase; AHAIR, acetohydroxy acid isomeroreductase; DHAD, dihydroxy acid dehydratase; TA-B, transaminase B; KMV, α-ketomethylvalerate; KIV, α-ketoisovalerate; KIV, α-ketoisocaproate

family of amino acids has been established (figure 3) and several differences to *E. coli* were observed. In contrast to *C. glutamicum*, *E.coli* possesses up to three isoenzymes for several of the enzymes part of the L-isoleucine synthesis, among them the aspartate kinase (Cohen *et al.*, 1969) and the AHAS (Umbarger, 1978), which makes the regulation even more complicated. As initial reaction into this metabolic branch, the aspartate kinase is feedback inhibited by L-lysine plus L-threonine (Cremer *et al.*, 1991). Therefore, many production strains for L-lysine and other amino acids of the aspartate family possess a feedback-resistant variant of the aspartate kinase. Also regulated are the enzymes of the branching point at aspartate semialdehyde. In contrast to *E. coli*, where the first enzyme of the L-lysine branch, the dihydrodipicolinat synthase, is inhibited by L-lysine (Yugari and Gilvarg, 1962), in *C. glutamicum* this enzyme is neither regulated on the enzymatic level nor in its gene expression (Cremer *et al.*, 1988). Here, only the HOM, leading into the L-threonine/L-isoleucine branch, underlies a regulation. This enzyme is allosterically inhibited in its activity by L-threonine (Eikmanns *et al.*, 1991; Miyajima et al., 1968), the expression of its gene is repressed by the presence of higher concentrations of L-methionine (Follettie *et al.*, 1988). Feedback-resistant variants of the HOM which are no longer inhibited by L-threonine are available and in use for the construction of L-threonine production

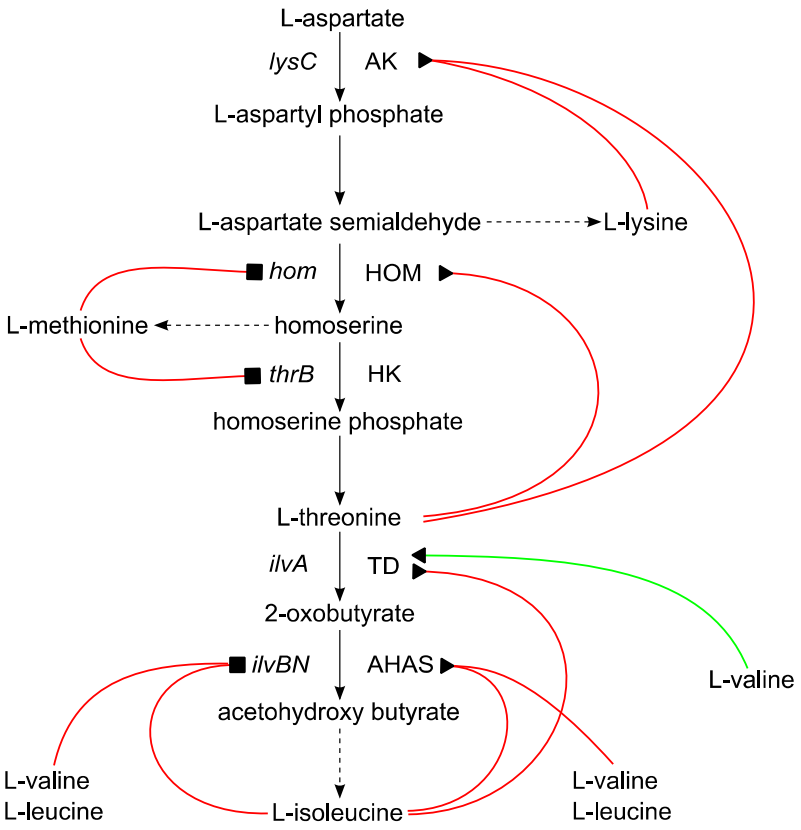

Figure 3: Regulation of the L-isoleucine biosynthesis in *C. glutamicum*. = repression; = inhibition; = activation.

strains (Reinscheid et al., 1991). The actual key enzymes of the L-isoleucine biosynthesis are the TD and the AHAS (figure 3). The TD as the only unique enzyme of the L-isoleucine synthesis is severely inhibited even by low concentrations of L-isoleucine. In contrast, L-valine has an activating effect on the TD (Möckel *et al.*, 1992). By these regulations the formation of 2-oxobutyrate is limited when sufficient L-isoleucine is present, while the formation of L-isoleucine is boosted when L-valine is present in excess. The AHAS is a hetero-tetramer consisting of two catalytic and two regulatory subunits. The genes of the two subunits, *ilvB* and *ilvN*, form an operon, that also comprises the gene of the AHAIR, *ilvC*. This operon is repressed by the presence of the three branched-chain amino acids L-isoleucine, L-valine and L-leucine by an attenuation mechanism (Morbach *et al.*, 2000). The presence of 2-oxobutyrate enhances the specific activity of the AHAS up to tenfold (Eggeling *et al.*, 1987; Morbach *et al.*, 2000), however, the mechanism of this activation is not known. Additionally, the AHAS is allosterically inhibited in its activity by the concerted action of L-isoleucine, L-valine and L-leucine. The simpler and more understandable regulation of this metabolic pathway in comparison with *E. coli* plus the absence of isoenzymes makes *C. glutamicum* the preferred organism for the biotechnological synthesis of amino acids of the aspartate family.

KIV production with *C. glutamicum*

Krause (2010) recently performed a project which had the aim to construct a KIV producer on the basis of *C. glutamicum* WT. The central aspects of strain construction were the deletion of the genes coding for the enzyme I of the PDHC (*aceE*-gene) and for the pyruvate:quinone oxidoreductase (*pqo*-gene) that led to an increase of the intracellular pyruvate pool, which in contrast to KMV is the single precursor of the KIV synthesis (figure 2). Furthermore *ilvE*, coding for the transaminase B, was deleted to prevent that KIV is converted to L-valine and the genes of the KIV synthesis pathway, *ilvBNCD*, were overexpressed. The strain showed a quite efficient KIV production from glucose as only carbon source. Another aspect which became obvious in the described work was that the AHAS as a key enzyme of the KIV and also the KMV synthesis is competitively inhibited by KIV and might also be influenced by KMV and KIC. The further characterization of the AHAS, especially focusing on the 2-oxobutyrate plus pyruvate reaction leading to KMV, also is of central interest in this work.

Tasks of the current work

The central aim of this work is a deeper understanding of the pathway leading to KMV and L-isoleucine and the rational construction of a production strain for KMV with *C. glutamicum*. The branched-chain α-keto acid KMV together with KIV and KIC may be used as substitute for their corresponding amino acids L-isoleucine, L-valine and L-leucine in medicine for patients who suffer from chronic renal diseases (Aparicio *et al.*, 2009; Chang *et al.*, 2009; Feiten *et al.*, 2005; Teschan *et al.*, 1998). The use of branched-chain α-keto acids instead of branched-chain amino acids reduces the nitrogen exposure for such patients. Another application for the branched chain α-keto acids is their use as ingredient in so-called "functional food", for instance drinks for sportsmen and body builders (Karau *et al.*, 2008). It has been described that KMV, KIV and KIC could improve the muscle regeneration after physical training. At the beginning of this work the production of the branched-chain α-keto acids was only possible by a set of chemical methods (Cooper *et al.*, 1983). A way for the biotechnological production of KMV with bacteria has not yet been described up to now. There are several studies that describe the design of efficient L-isoleucine production strains in *C. glutamicum* (Morbach *et al.*, 1995 + 1996; Kelle *et al.*, 1996). Many of those strains were constructed by modification of production strains that were made by undirected mutagenesis. In contrast to that, the aim of this work is the construction of a genetically defined production strain for KMV. The strain design will follow a similar approach as some of the described L-isoleucine-producers by using a L-lysine producer as a basis, but the respective strain will be one that was obtained by rational design. Another subject will be the research of the behavior of *C. glutamicum* in the presence of branched-chain α-keto acids. Furthermore key enzymes of the KMV-synthesis such as the TD and the AHAS will have to be analyzed on their activity in the presence of high concentrations of KMV.

2. Material and Methods

2.1 Strains, plasmids and oligonucleotides

Bacterial strains

Strain	Characteristics	Reference
C. glutamicum ATCC 13032	wild type (WT)	Abe *et al.*, 1967
C. glutamicum Δ*ilvE*	*C. glutamicum* WT with deletion of *ilvE*	Marienhagen *et al.*, 2005
C. glutamicum Δ*ilvA* Δ*panBC*	*C. glutamicum* WT with deletion of *ilvA* and *panBC*	Radmacher *et al.*, 2002
C. glutamicum ins*lpd*	*C. glutamicum* WT with insertion in *lpd*	L. Eggeling, Forschungszentrum Jülich
C. glutamicum Δ*odhA*	*C. glutamicum* WT with deletion of *odhA*	Hoffelder et al., 2010
C. glutamicum Δ*ilvA*	*C. glutamicum* WT with deletion of *ilvA*	Sahm and Eggeling, 1999
C. glutamicum DM1729	*C. glutamicum* WT with *pyc*P458S, *hom*V59A, *lysC*T311I	B. Bathe, Evonik AG
C. glutamicum DM1729 Δ*ilvE*	*C. glutamicum* DM1729 with deletion of *ilvE*	This work
C. glutamicum DM1729 Δ*ilvE* *dapA*-Prom:C5	*C. glutamicum* DM1729 Δ*ilvE* with exchange of *dapA* promoter against C5 promoter	This work
C. glutamicum DM1729 Δ*ilvE* *dapA*-Prom:C13	*C. glutamicum* DM1729 Δ*ilvE* with exchange of *dapA* promoter against C13 promoter	This work
C. glutamicum DM1729 Δ*ilvE* *dapA*-Prom:B31	*C. glutamicum* DM1729 Δ*ilvE* with exchange of *dapA* promoter against B31 promoter	This work
E. coli DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
E. coli XL1-Blue	F' ::Tn10 *proA*$^{+}$*B*$^{+}$ *lacI*^{q} Δ(*lacZ*)M15/*recA*1 *endA*1 *gyrA*96(*Nal*^{r})thihsdR17 (r_K- m_K+) *glnV*44 *relA*1 *lac*	Bullock *et al.*, 1987

Plasmids

Plasmid	Characteristics	Reference
pK19mobsacB ilvE-del	pK19mobsacB with truncated *ilvE*	Marienhagen *et al.*, 2005
pEK *hom*^{FBR} *thrBthrC*	*E. coli* / *C. glutamicum* shuttle vector pEK0: Km^R	Eikmanns *et al.*, 1991
pMM36ptac	*E. coli* / *C. glutamicum* shuttle vector: ptac-promoter, Cm^R	Krause *et al.*, 2010
pMM36ptac *ilvA*	pMM36ptac expressing *ilvA* with upstream *gap* RBS	This work
pMM36ptac *tdcB*	pMM36ptac expressing *tdcB* with upstream *gap* RBS	This work
pECM3 *ilvA*V323A	*E. coli* / *C. glutamicum* shuttle vector: Cm^R, expressing feedback-resistant *ilvA*	Möckel *et al.*, 1994
pECM3 *ilvA*D378G	*E. coli* / *C. glutamicum* shuttle vector: Cm^R, expressing feedback-resistant *ilvA*	Möckel *et al.*, 1994
pECM3 *ilvA*H278RL351S	*E. coli* / *C. glutamicum* shuttle vector: Cm^R, expressing feedback-resistant *ilvA*	Möckel *et al.*, 1994
pK19mobsacB	*C. glutamicum* integration vector: Km^R, lacZα, *sacB*-gene for selection of double cross-over events	Schäfer *et al.*, 1994
pK19mobsacB *dapA*-Prom:C5	pK19mobsacB with modified *dapA*-promoter	Hell, 2010
pK19mobsacB *dapA*-Prom:C13	pK19mobsacB with modified *dapA*-promoter	Hell, 2010
pK19mobsacB *dapA*-Prom:B31	pK19mobsacB with modified *dapA*-promoter	Hell, 2010

Oligonucleotides

Oligonucleotide	Sequence	Purpose
ilvAfor-gapRBS SmaI	TCCCCCGGGAGGAGACACACCATGAGTGAAACATA CGTGTCT	Expression of *ilvA* in pMM36ptac
ilvArev StuI	GAAGGCCTTTAGGTCAAGTATTCGTACTCAG	Expression of *ilvA* in pMM36ptac
ptac seq	TCAAGGCGCACTCCCGTTCT	Sequencing primer for pMM36ptac
ilvA seq mid	GCACAATGGTGGACCAATC	Sequencing primer for *ilvA*
ilvA seq rev	AGGAAGTGACGCAACTGAC	Sequencing primer for *ilvA*
pMM36_rev	GCCAGTTTGCTCAGGCTCTC	Sequencing primer for pMM36ptac
tdcB (*E. coli*) hin gapRBS	AGGAGACACAACATGCATATTACATACGATCTGCC GGTTGC	Expression of *tdcB* in pMM36ptac
tdcB (*E. coli*) rück PstI	AACTGCAGAACCTTAAGCGTCAACGAAACCGGTG	Expression of *tdcB* in pMM36ptac
ilvE 1	GCGTTGACTGATTCTTGGTC	Proof of *ilvE*-deletion
ilvE 2	CGAGTTCGATGGAATCTTCG	Proof of *ilvE*-deletion
dapA hin NdeI	TGGTCCAGGTCCAGGGAATTCCATATGAGCACAGG TTTAACAGC	Flanking region for *dapA*-promoter exchange
dapA rück BamHI	CGCGGATCCACCGACCAGGAATGTCATAG	Flanking region for *dapA*-promoter exchange
thyX hin XbaI	CTAGTCTAGAGCGGAGAATCGGAAGTAGTG	Flanking region for *dapA*-promoter exchange
thyX rück NsiI	GAATTCCCTGGACCTGGACCAATGCATGAACAATT GACACGAGCAAG	Flanking region for *dapA*-promoter exchange
dapA-Prom hin NsiI kurz	CCAATGCATGCAGTCTTGACCTTCCTGTTATCGGA ATGTGGCTTGGGCGATTG	Amplification of *dapA*-promoter
dapA-Prom C5 rück NdeI	GGGAATTCCATATGGTTCAAGGTTACAATCTTCCC TCATTTG	Amplification of *dapA*-promoter with modifications
dapA-Prom C13 rück NdeI	GGGAATTCCATATGGTTCAAGGTTACCGTCTTCCC TCATTTG	Amplification of *dapA*-promoter with modifications
dapA-Prom B31 rück NdeI	GGGAATTCCATATGGTTCAAGGTTCCCTTCTTCCC TCATTTG	Amplification of *dapA*-promoter with modifications
dapA-Prom Nachweis	GCAGTCTAGACGTTCCTG	Proof of *dapA*-promoter exchange in *C. glutamicum*

2.2 Chemicals and equipment

Chemicals

The following chemicals were used in this work:

Acetic acid, 100 % anhydrous	Merck KGaA, Darmstadt
Agarose	Gibco BRL Life Technologies, Inc., Eggenstein
Aspartate sodium salt monohydrate	Fluka Chemie GmbH, Buchs, Switzerland
ATP	Roche Diagnostics GmbH, Mannheim
Ammonium sulfate	Fluka Chemie GmbH, Buchs, Switzerland
Bacto tryptone	BD (Becton, Dickinson and Co.), Sparks, USA
Bacto yeast extract	BD (Becton, Dickinson and Co.), Sparks, USA
d-biotine (vitamine H)	Sigma-Aldrich Chemie GmbH, Steinheim
Brain-Heart-Infusion (BHI)	Difco Laboratories, Augsburg
Calcium chloride dihydrate	Merck KGaA, Darmstadt
Chloroform	Merck KGaA, Darmstadt
DDB (1,2-diamino-4,5-dimethoxy benzole)	Eugene, Leiden, the Netherlands
Desoxynucleotide triphosphate mix	MBI Fermentas GmbH, St. Leon-Rot
Dithiothreitol (DTT)	BTS-Biotech Trade % Service Laborbedarf GmbH, St. Leon-Rot
EDTA disodium salt dihydrate	AppliChem GmbH, Darmstadt
Ethanol, absolute puriss.	Merck KGaA, Darmstadt
Ethanol, absolute denatured	Merck KGaA, Darmstadt
Glass beads, acid washed	Sigma-Aldrich Chemie GmbH, Steinheim
Glycerol	Carl Roth GmbH & Co. KG, Karlsruhe
Hydrochloric acid, 25 %	Merck KGaA, Darmstadt
Isoamyl alcohol	Merck KGaA, Darmstadt
Isopropanol	Merck KGaA, Darmstadt
Iron(II) sulfate	Merck KGaA, Darmstadt
Kanamycin	Merck KGaA, Darmstadt
2-ketoisocaproate sodium salt	Sigma-Aldrich Chemie GmbH, Steinheim
2-ketoisovalerate sodium salt	Sigma-Aldrich Chemie GmbH, Steinheim

2-ketomethylvalerate sodium salt	Sigma-Aldrich Chemie GmbH, Steinheim
2-ketovalerate sodium salt	Sigma-Aldrich Chemie GmbH, Steinheim
Magnesium chloride hexahydrate	Merck KGaA, Darmstadt
Magnesium sulfate heptahydrate	Sigma-Aldrich Chemie GmbH, Steinheim
β-mercapto ethanol	Serva Feinbiochemika, Heidelberg
Morpholino propanesulfonic acid (MOPS)	Carl Roth GmbH & Co. KG, Karlsruhe
NAD$^+$ disodium salt	Gerbu Biotechnik GmbH, Gaisberg
NADH + H$^+$ disodium salt	Gerbu Biotechnik GmbH, Gaisberg
NADPH + H$^+$ disodium salt	Gerbu Biotechnik GmbH, Gaisberg
Nickel chloride hexahydrate	Sigma-Aldrich Chemie GmbH, Steinheim
Phenol	Carl Roth GmbH & Co. KG, Karlsruhe
Potassium acetate	Merck KGaA, Darmstadt
Potassium chloride	Merck KGaA, Darmstadt
Potassium dihydrogen phosphate	Merck KGaA, Darmstadt
di-potassium hydrogen phosphate	Merck KGaA, Darmstadt
Potassium hydroxide	Merck KGaA, Darmstadt
Pyruvate disodium salt	Fluka Chemie GmbH, Buchs, Switzerland
Semicarbazide hydrochloride	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium acetate	Merck KGaA, Darmstadt
Sodium chloride	AppliChem GmbH, Darmstadt
Sodium dodecyl sulfate (SDS)	Fluka Chemie GmbH, Buchs, Switzerland
Sodium hydroxide	AppliChem GmbH, Darmstadt
Sulfuris acid, 95 – 97 %	Merck KGaA, Darmstadt
Threonine	Fluka Chemie GmbH, Buchs, Switzerland
Triethanolamine	Merck KGaA, Darmstadt
Tris base	Sigma-Aldrich Chemie GmbH, Steinheim
Zinc sulfate heptahydrate	Merck KGaA, Darmstadt

Equipment

Incubation shakers

• Certomat MO	B. Braun Biotech International, Melsungen
• G 24 environmental Incubator Shaker	New Brunswick Scientific Inc., Nürtingen

Photometer

• Ultrospec 3000	Amersham Pharmacia Biotech GmbH, Freiburg
• Ultrospec 2100 pro	Amersham Pharmacia Biotech GmbH, Freiburg

Chromatography

• LC 1100 HPLC device with fluorescence (G1321A) and UV detector (G1314B)	Agilent Technologies Deutschland GmbH, Böblingen
• Precolumn: C18-Multohyp, ODS-5µ, 40×4 mm	CS-Chromatographie Service GmbH, Langerwehe
• Main column: C18-Multohyp, ODS-5µ, 125×4 mm	CS-Chromatographie Service GmbH, Langerwehe
• Precolumn: Organic Acid, 40×8 mm	CS-Chromatographie Service GmbH, Langerwehe
• Main column: Organic Acid, 300×8 mm	CS-Chromatographie Service GmbH, Langerwehe
• Chrompack CP9001 Gas chromatograph	Varian Deutschland GmbH, Darmstadt
• Autosampler for gas chromatograph	CTC Analytics AS, Zwingen (Switzerland)
• Hamilton syringe for gas chromatograph	Hamilton Bonaduz AG, Bonaduz (Switzerland)

Balances

• Sartorius BP 8199	Sartorius AG, Göttingen
• Sartorius BP 2100	Sartorius AG, Göttingen

Centrifuges

• L8-60M ultra centrifuge	Beckmann, USA

- Zentrifuge 5804 R — Eppendorf-Netheler-Hinz GmbH, Köln
- Heraeus Sepatech Minifuge RF — Heraeus Holding GmbH, Stuttgart
- Galaxy 14D — VWR international, Darmstadt
- Sigma 202 MK — Sigma, Osterode am Harz

Further equipment

- Anthos hat III Platereader — Anthos Microsysteme GmbH, Krefeld
- Photo documentation device — Decon Science Tec GmbH, Hohengnadern
- Gene pulser — Bio-Rad Laboratories GmbH, München
- PCR cycler — Biometra GmbH, Göttingen
- pH meter WTW pH521 — Wissenschaftlich-Technische Werkstätten, Weilheim
- RiboLyser™ — Thermo Hybaid GmbH, Garching
- UV screen — Bachhofer, Reutlingen

Enzymes

All enzymes used in this work are listed below:

- Alkaline phosphatase — MBI Fermentas GmbH, St. Leon-Rot
- Hexokinase/glucose-6-phosphate DH — Roche Diagnostics GmbH, München
- Lysozyme — Roche Diagnostics GmbH, München
- Proteinase K — Roche Diagnostics GmbH, München
- *Pwo* DNA polymerase — PeqLab Biotechnologie GmbH, Erlangen
- Restriction enzymes — MBI Fermentas GmbH, St. Leon-Rot
- RNase A (DNase free) — MBI Fermentas GmbH, St. Leon-Rot
- T4 DNA ligase — MBI Fermentas GmbH, St. Leon-Rot
- *Taq* DNA polymerase — Genaxxon BioScience GmbH, Ulm

Kits

All Kits used in this work for protein determination and isolation and clean up of nucleic acids are listed below:

- BCA protein assay — Thermo Scientific, Rockford, USA
- E.Z.N.A. Plasmid Miniprep Kit I — Omega Bio-Tek Inc., Norcross, USA
- NucleoSpin Extract II — Machery-Nagel GmbH & Co. KG, Düren
- Perfectprep Plasmid Mini — Eppendorf AG, Hamburg

2.3 Nutrient solutions and culture conditions

The following nutrient solutions were used for the cultivation of *C. glutamicum* and *E. coli* strains. For the production of agar plates agar (18 g/l) was added to the media.

2×TY-complex medium	Tryptone	16 g/l
(Sambrook *et al.*, 2001)	Yeast extract	10 g/l
	NaCl	5 g/l
BHI-complex medium	brain-heart-infusion powder	37 g/l
BHIS	brain-heart-infusion powder	37 g/l
(Liebl *et al.*, 1989)	Sorbitol	91 g/l
CgXII-minimal medium	$(NH_2)_2SO_4$	20 g/l
(modified after Kase *et al.*,	Urea	5 g/l
1972)	3-(N-morpholino)propanesulfonic acid	21 g/l
	K_2HPO_4	5 g/l
	KH_2PO_4	5 g/l
	$MgSO_4 \times 7\ H_2O$	0.25 g/l
	$CaCl_2$	0.01 g/l

The pH of the solution was set to 6.8 by the addition of KOH. After autoclaving 1 ml/l of the following solutions was added to the medium:

Micronutrient solution (1000×),

Biotin (200 mg / ml)

As a carbon source glucose was added to the medium with concentrations of 1 % (w/v) up to 4 % (w/v).

Micronutrient solution (1000×)	$FeSO_4 \times 7\ H_2O$	16.4 g/l
	$MnSO_4 \times H_2O$	10.0 g/l
	$CuSO_4 \times 5\ H_2O$	0.2 g/l
	$ZnSO_4 \times 7\ H_2O$	1.0 g/l
	$NiCl_2 \times 6\ H_2O$	0.02 g/l

The salts were solved in water with the addition of concentrated HCl. The solution was sterile filtrated and kept in a closed glass flask under N_2 atmosphere.

Culture conditions

For growth experiments, at first 5 ml 2×TY-complex medium were inoculated with a single colony of the respective *C. glutamicum* strain and incubated on a rotary shaker at 120 rpm and 30 °C. The 5 ml culture were used to inoculate a 50 ml 2×TY preculture, which was incubated over night at 120 rpm and 30 °C. The overnight culture served as inoculants for the main culture consisting either of 50 ml 2×TY-complex medium or of 50 ml CgXII-minimal medium in baffled 500 ml Erlenmeier flasks. For this purpose the preculture was sedimented by centrifugation and washed twice with 0.9 % NaCl-solution. The cells were suspended in 1 ml NaCl-solution. An amount required to get a starting optical density at 600 nm wavelength (OD_{600}) of 1 or 1.5 in the main culture was taken for inoculation. The cultivation was performed on a rotary shaker at 120 rpm and 30 °C and the development of the growth was followed by measurement of the OD_{600}.

Preparation of glycerol cultures

To store strains for a longer period of time, they were kept in glycerol cultures at – 70 °C. For this purpose 5 ml 2×TY-complex medium were inoculated with a single colony and incubated over night on a rotary shaker at 120 rpm. 700 µl of the grown culture were mixed with 300 µl of sterile glycerol in a 1.5 ml screw cap tube and stored at -70 °C.

2.4 Isolation of nucleic acids

The following solutions are required for the preparation of plasmid or chromosomal DNA:

Solution A:	50 mM glucose
	25 mM Tris
	10 mM EDTA
	➔ pH 8.0 with HCl
Solution B:	0.2 M NaOH
	1 % SDS
Solution C:	3 M potassium acetate
	11.5 % acetic acid
TE-buffer:	10 mM Tris
	1 mM EDTA
	➔ pH 7.6 with HCl
lysis buffer:	10 mM Tris
	400 mM NaCl
	2 mM EDTA
	➔ pH 8.2 with HCl

Mini plasmid preparation from *E. coli*

The isolation of small amounts of plasmid DNA from *E. coli* was done using the method of alkaline lysis of cells (Birnboim *et al.*, 1983). Depending on the density between 1.5 and 5 ml of a culture grown in 2×TY-complex medium was sedimented by centrifugation for 30 sec at 8000 rpm. The pellet was suspended in 100 µl of solution A and incubated for 5 min at room temperature. Thereafter 200 µl of freshly prepared solution B were added, mixed and incubated for 5 min on ice. At this the alkaline cell lysis takes place, whereupon the unleashed proteins and nucleic acids are degraded because of the basic pH. To restore the native form of the nucleic acids 150 µl of the sour solution C were added, carefully mixed and again incubated for 5 min on ice. The denaturated proteins and all other solid cell parts were pelletized via centrifugation. Proteins left in the supernatant were removed by phenol/chloroform extraction. The plasmid-DNA was precipitated by addition of 1 ml ice cold absolute ethanol and centrifugation at 10,000 rpm for 10 min and washed in 70 % ethanol afterwards. The dried pellet was suspended in demineralized water.

Alternatively a plasmid mini preparation kit was used for the isolation of plasmids from *E. coli*.

Midi plasmid preparation from *E. coli*

To isolate larger amounts of a plasmid a midi plasmid preparation was carried out (Sambrook *et al.*, 2001), which also works after the principal of alkaline lysis. For this the *E. coli* strain harboring the respective plasmid was grown in 50 ml 2×TY-complex medium over night. The pelletized cells were suspended in 5 ml solution A with the addition of a spatula tip of lysozyme to facilitate a better cell lysis. After a 10-minute incubation at room temperature 10 ml of solution B and after another 10 minutes on ice, 7.5 ml of solution C were added. The mixture was again incubated for 10 minutes on ice and afterwards the solid cell components were sedimented by centrifugation at 5,000 rpm and 4 °C for 30 min. The supernatant was filtered through mull into a fresh 50 ml falcon tube. The DNA was precipitated by addition of 12 ml isopropanol and another 10 min of centrifugation. The supernatant was discarded and the pellet was dried at 37 °C to evaporate the remaining isopropanol. The pellet was solved in 2 ml of demineralized water. To remove the contained RNA, 2 ml of a 5 M LiCl / 50 mM Tris/HCl (pH 7.5) solution was added and the solution was incubated for 10 min on ice. The precipitated RNA was removed by centrifugation and the supernatant was transferred into a fresh reaction tube. Again the DNA was precipitated by addition of 10 ml ethanol and incubation over night at – 20 °C. The solution was centrifuged and the pellet was resolved in 400 µl 10 mM Tris/HCl (pH 7.6) solution. To get rid of remaining RNA, 5 µl RNase A (10 mg/ml) was added and the sample was

incubated for 30 min at 37 °C. Afterwards a phenol / chloroform extraction was carried out to remove the RNase together with other remained proteins. Afterwards the plasmid DNA was precipitated another time by addition of 1 ml ice cold absolute ethanol and incubation for 2 h at – 20 °C followed by a centrifugation step at 13,000 rpm and 4 °C for 15 min. The pellet was washed with 70 % ethanol, dried at 37 °C and resolved in demineralized water.

Mini plasmid preparation from *C. glutamicum*

The mini plasmid preparation from *C. glutamicum* was made out of 5 ml of a 2×TY overnight culture. The cells were sedimented via centrifugation and washed once in 1 ml 0.9 % NaCl solution. The pellet was solved in 200 µl of solution A and a spatula tip of lysozyme was added to facilitate the cell lysis. The mixture was incubate for 1 -2 hours at 37 °C after which 400 µl of freshly prepared solution B were added, carefully mixed and incubated on ice for 5 min. Then 350 µl of solution C were added and again incubated for 5 min on ice. The solid cell fragments were pelletized by centrifugation with 13,00o rpm and 4 °C for 10 min and the supernatant which contains the plasmid DNA was cleaned via phenol-chloroform extraction. Afterwards the DNA was precipitated by the addition of 250 µl isopropanol and 25 µl of solution C. After 10 min of centrifugation the pellet was washed in 500 µl of 70 % ethanol, centrifuged again and the pellet was dried. At last the pellet was suspended in 30 µl of demineralized water.

Isolation of chromosomal DNA from *C. glutamicum*

The isolation of chromosomal DNA, which is used as template for PCR, was carried out using the protocol of Eikmanns *et al.*, (1994). Therefore, 5 ml of a *C. glutamicum* culture grown over night were pelletized by centrifugation, washed twice in TE-buffer and solved again in 1 ml TE-buffer with 15 mg/ml lysozyme. After 3 h of incubation at 37 °C, 3 ml of lysis-buffer and 150 µl proteinase K solution (20 mg/ml) were added and further incubated over night. The contained proteins were precipitated by addition of 2 ml saturated NaCl-solution and afterwards sedimented via centrifugation. By addition of ice cold ethanol the DNA in the supernatant precipitates as visible threads, which were fished with a Pasteur pipette formed to a hook by bending on the flame of a Bunsen burner. The chromosomal DNA was washed in 70 % ethanol, dried shortly in the air and finally suspended in demineralized water.

2.5 Clean up of nucleic acid solutions

NucleoSpin

To clean plasmids and PCR products from contained protein and to change buffers the NucleoSpin extraction kit was used. The clean up was performed as described in the manufacturer's manual. The DNA was eluted from the columns with an appropriate amount of demineralized water. The NucleoSpin kit was also used for the extraction of restriction digested DNA-fragments from an agarose gel.

Ethanol precipitation

To change the buffer of a nucleic acid solution the method of ethanol precipitation was applied. For this 0.1 volumes of a 3 M sodium acetate solution (pH 5.2) and 2.5 volumes of ice cold absolute ethanol were added to the sample and incubated for 2 hours at -20 °C. The precipitated DNA was sedimented by centrifugation at 13,000 rpm at 4 °C for 10 min. The pellet was washed with 70 % ethanol, centrifuged again and dried at the air to get rid of remaining ethanol. At last the pellet was solved in an appropriate amount of demineralized water.

Phenol-chloroform extraction

To clean nucleic acid solutions especially plasmid preparations from contained protein a phenol-chloroform extraction was applied. The respective solution was mixed with 1 volume of a phenol/chloroform/isoamyl alcohol solution (25:24:1 [v/v/v]) by heavy shaking. Centrifugation with 13,000 rpm at room temperature for 5 min leads to a division in two phases, a lower phenolic phase and an upper watery phase that contains the nucleic acids. Protein accumulates as an interphase between the two phases. The upper phase was transferred into a new reaction tube, while the lower phase was discarded. This procedure was repeated as long as there was a visible protein interphase. To get rid of remaining phenol the sample was mixed with a chloroform/isoamyl alcohol solution (24:1 [v/v]) and centrifuged again which results in another phase division. The upper watery phase that contains the DNA was transferred into a new reaction tube and the DNA was precipitated by the addition of ethanol.

2.6 Transformation of *C. glutamicum* and *E. coli* cells

Preparation of competent *C. glutamicum* cells

C. glutamicum as a Gram-positive organism is more difficult to be transformed in comparison to many Gram-negative bacteria due to the thicker mureine layer. Therefore a special protocol has to be applied to obtain *C. glutamicum* cells which are more competent for electroporation. This method is based on the effect that very fast growing bacteria cannot develop their cell wall as dense as bacteria growing with a lower growth rate. A fast growth is allowed by cultivation in the rich complex medium BHIS.

In the actual protocol used in this work 250 ml prewarmed BHIS medium in 1 l baffled Erlenmeyer flasks were inoculated with 5 ml of a BHIS preculture and cultured at 30 °C on a rotary shaker until an OD_{600} of 1.75 was reached. Then the culture was divided into 5 cooled 50 ml Falcon tubes and the cells were pelleted by centrifugation at 5,000 rpm and 4 °C for 15 min. All following steps were performed on ice, the centrifugation steps at 4 °C. The pellets were suspended in 20 ml ice cold TG-buffer (1 mM Tris/HCl, pH 7.5; 10 % [v/v] glycerol) and washed twice in this buffer. After the first washing step, the cells were unified in two Falcon tubes. This was followed by two further washing steps in ice cold 10 % [v/v] glycerol solution. The pellets were then suspended in 1 ml 10 % [v/v] glycerol and aliquoted in 150 µl portions. Those samples were frozen in liquid nitrogen and stored at – 70 °C until further use.

Preparation of competent *E. coli* cells

For the preparation of electrocompetent *E. coli* cells, 250 ml LB-complex medium in a 1 l baffled Erlenmeyer flask was inoculated with 500 µl of a preculture grown over night. The culture was incubated on a rotary shaker until an OD_{600} between 0.3 and 0.5 was reached. At this point the culture was harvested, divided into cooled 50 ml Falcon tubes and incubated for 30 min on ice. All following steps were performed on ice, the centrifugation steps at 4 °C. The cells were sedimented by 15 min of centrifugation at 5,000 rpm and afterwards suspended in 250 ml 10 % [v/v] glycerol. After another centrifugation step the pellets were solved in 100 ml 10 % [v/v] glycerol and unified in two Falcon tubes. Again the suspension was centrifuged and another washing step with 25 ml 10 % [v/v] glycerol followed. At last the pellets were suspended in 500 µl 10 % [v/v] glycerol and aliquoted in 50 µl portions in Eppendorf cups. The aliquots were frozen in liquid nitrogen and stored at – 70 °C until further use.

Transformation by electroporation

For the transformation of *C. glutamicum* via electroporation appropriate competent cells were thawed on ice. The competent cells were mixed with 0.1 – 10 µg plasmid DNA in a cooled Gene-Pulser cuvette (0.2 cm electrode distance), which were sterilized on a UV-screen previously. The mixture was incubated on ice for 10 min before the electroporation. The transformation took place in a Gene-Pulser device with the settings of 2.5 KV, 25 µF and 200 Ω. After the electric pulse the cells were transferred into 5 ml prewarmed BHIS medium and incubated for 6 min at 46 °C in a water bath. Afterwards the cells were regenerated by incubation on a rotary shaker at 30 °C for 50 min. During this time the successfully transformed cells can develop the antibiotic resistance which is encoded on the plasmid. At last the cells are plated on selective agar plates that contain the respective antibiotics and incubated at 30 °C.

The transformation of *E. coli* is performed with a similar protocol as the transformation of *C. glutamicum*. The mixture of competent cells and plasmid DNA was not preincubated, but the electric pulse was applied directly after the mixing. For the electroporation the same settings were used as with *C. glutamicum*. The cells were transferred in 200 µl 2×TY complex medium and regenerated at 37 °C for 50 min. For the transformation of *E. coli* no heat shock was necessary. At last the cells are plated on selective agar plates that contain the respective antibiotics and incubated at 37 °C.

2.7 Enzymatic treatment of nucleic acids

Polymerase chain reaction

The polymerase chain reaction (PCR; Saiki *et al.*, 1988) is the standard method for the specific amplification of DNA fragments by a DNA polymerase. Two primer molecules that specifically hybridize with the template DNA mark the beginning and end of the amplified fragment. The DNA polymerase adds new nucleotides complementary to the template strain on the 3`-OH end of the primers and on this way synthesizes a double stranded DNA fragment from a single stranded template. A typical PCR assay of 50 µl contained 0.1 – 1 µg of chromosomal DNA as a template, the two specific PCR primers (0.3 µM each), dNTPs (200 µM each), a reaction buffer and 6 % [v/v] DMSO which improves the denaturation of high GC templates. As final component 1 U of a *Taq* DNA polymerase was added to the assay. The typical PCR program started with an

initial denaturation step at 95 °C for 5 min to divide the template into single stranded molecules. The following denaturation step (30 sec at 95 °C), the alignment step (30 sec) and the elongation step (72 °C) were repeated in cycles between 30 and 35 times. The temperature of the alignment step was dependent on the melting temperature of the used primers and was usually chosen in the range between 45 °C and 60 °C. The duration of the elongation step in which the DNA polymerase synthesizes the new strain was dependent on the length of the amplified fragment. The *Taq* DNA polymerase needs 1 min for the synthesis of a 1 kb fragment. The program ended with a final elongation step for 5 min at 72 °C to ensure the complete synthesis of double stranded products in full length.

DNA fragments intended for plasmid constructions were amplified using a DNA polymerase with proofreading capability. Therefore the assay mixture and the PCR program were adapted as described in the manufacturer's manual.

Restriction digest

Restriction digestion of DNA was performed using enzymes of MBI-Fermentas GmbH. The assays were prepared as described in the manufacturer`s manual. Analytical digestions were made in 20 µl assays, for preparative applications a 50 or 100 µl scale was chosen. After stopping the digestion reactions by a heat shock at 65 or 80 °C (depending on enzyme), the success of the digestion was controlled via agarose gel electrophoresis.

Ligation of PCR fragments with plasmid DNA

Both the plasmid and the insert were restriction digested before the ligation with enzymes providing compatible ends. Additionally the linearized plasmid was treated with alkaline phosphatase to prevent religation. A ligation assay contained 1 µl T4 DNA ligase (5 U / µl), ligation buffer, the plasmid and insert DNA in a total volume of 20 µl. To improve the chances for a successful ligation at least three assays were made with plasmid and insert in different proportions. In the case of a blunt-end ligation hexamine cobalt chloride was added to the assay to improve the interaction of free DNA ends. The assays were incubated for 90 min at 22 °C or alternatively over night at 16 °C. The reaction was stopped by heating to 65 °C. Afterwards the ligation was transformed into *E. coli* via electroporation.

2.8 Agarose gel electrophoresis

The following solutions are required for an agarose gel electrophoresis:

50 × TAE buffer:	2 M Tris / HCl, pH 8.0
	50 mM EDTA
	500 mM sodium acetate
loading dye:	0.25 % bromophenol blue
	40 % glycerol

Agarose gel electrophoresis was applied for the analytical and the preparative separation of DNA fragments. Due to the negative charge of the phosphate groups of nucleic acids, DNA fragments move towards the anode in an electric field. The agarose gel forms a molecular web that allows smaller fragments to move faster than longer DNA molecules and therefore they are separated by their length (Sambrook *et al.*, 2001).

In most cases 0.8 % [w/v] agarose was used, for fragments bellow 300 bps a 2 % [w/v] agarose solution was prepared that allows a better separation of small DNA molecules due to the tighter web. The agarose was mixed with TAE buffer and solved by heating in a microwave. The solution was cooled to about 60 °C and casted into the prepared gel chamber. TAE buffer was used as the running buffer. The samples were mixed with 0.25 volumes of loading dye and pipetted into the pockets of the gel. For size determination an appropriate size marker was pipette into another pocket. The separation was performed at 90 V. Afterwards the gel was stained in a watery ethidium bromide solution (1 µg/ml). The fluorescent dye Ethidium bromide intercalates into the DNA so that it can be made visible on a UV-screen (λ = 312 nm) and photographs can be made.

2.9 Determination of glucose concentration

To follow the substrate consumption in growth experiments the glucose concentration in culture supernatants was measured via an enzymatic assay. Glucose is phosphorylated by the hexokinase with ATP to glucose-6-phosphate which is afterwards converted to 6-phospho gluconate by the glucose-6-phosphate dehydrogenase. In the second reaction step the cosubstrate $NADP^+$ is reduced to $NADPH + H^+$ in stoichiometric equivalent amounts to the conversion of glucose. The formation of $NADPH + H^+$ was photometrically detected at 365 nm wavelength. In the following paragraph the required solution and the composition of a typical reaction assay is described:

Reaction buffer: 0.4 M Tris / HCl, pH 7.6

4 mM $MgSO_4$

Glucose determination assay: 500 µl reaction buffer

100 µl $NADP^+$ solution (4.4 mg / ml)

100 µl ATP solution (9.6 mg / ml)

100 µl sample (max. 1.65 mM glucose)

190 µl H_2O

The reaction assay was mixed and the extinction at 365 nm wavelength was measured (E1). To start the reaction 10 µl of enzyme mixture (hexokinase and glucose-6-phosphate dehydrogenase) was added to the assay and incubated at room temperature for 10 min. Afterwards the extinction at 365 nm was measured again (E2). The glucose concentration was calculated with the following formula:

$$c = \frac{V \times (E2 - E1)}{\varepsilon \times g \times v}$$

c = glucose concentration

V = total assay volume

ε = extinction factor of NADPH + H+ at 365 nm wavelength (3.4 $(mmol * cm)^{-1}$)

g = gauge of the cuvette (1 cm)

v = sample volume

2.10 Measurement of enzyme activities

Cell disruption

To prepare cell extracts for enzyme assays cell pellets of 50 ml cultures were disrupted mechanically using a RiboLyser™. Therefore the pellets were solved in 1 ml of a disruption buffer described in the respective enzyme assay protocol. The cell suspension was filled in 1.5 ml screw cap reaction tubes that contained 250 µl glass beads. If not described otherwise in the enzyme assay protocol, the disruption with the RiboLyser™ was performed in five steps of 20 seconds at level 6.5 (highest level). Between the disruption steps the samples had to be cooled on ice for five minutes because of the frictional heat. Afterwards the samples were centrifuged at 4 °C and 14,000 rpm for 20 min to get rid of solid cell fragments and glass beads. The supernatant was used as cell extract in the enzyme assays.

Determination of protein concentration

To calculate the specific activity of an enzyme it is necessary to know the protein concentration of the cell extract. All protein measurements were performed using the "BCA protein assay" of Thermo Scientific (Rockford, USA). The cell extracts were diluted in an appropriate manner to achieve a concentration in the detection range of the test. The test was made as described in the manufacturer's instructions. Finally the extinction of the samples at 562 nm was read using an Anthos ht III platereader.

Calculation of enzyme activities

Specific enzyme activities were calculated using the following formula:

$$\text{Specific activity [U/mg protein]} = \frac{\Delta E/\text{min} * \text{volume}}{\varepsilon * g * \text{sample volume} * \text{protein concentration}}$$

ΔE/min = extinction change per minute

volume = total volume of the test assay

ε = extinction factor

g = gauge of the cuvette (1 cm)

sample volume = volume of the used cell extract

α-oxo-acid dehydrogenase assay

The following paragraph describes the measurement of the activity of the pyruvate dehydrogenase complex (PDHC), the 2-oxoglutarate dehydrogenase complex (ODHC) and a branched-chain α-keto-acid dehydrogenase complex (BKDHC). The PDHC catalyzes the oxidative decarboxylation of pyruvate under the formation of acetyl-CoA and NADH + H^+. The ODHC performs the analogous decarboxylation of 2-oxoglutarate to succinyl-CoA. The BKDHC decarboxylates KMV, KIV and KIC. The test was performed as described by Creaghan and Guest (1972). The following solutions are required for the preparation of cell extract:

TES washing buffer: 100 mM TES
10 mM $MgCl_2$
3 mM cysteine
➔ pH 7.2

TES disruption buffer: TES washing buffer + 30 % [v/v] glycerol

The cells for the test were cultured in 2×TY-complex medium up to an OD_{600} of 3 to 4 or in CgXII-minimal medium up to an OD_{600} of 18. The harvested cells were washed twice in TES washing buffer and stored at -20 °C until further use. For the preparation of cell extract, the cell pellets were suspended in TES-disruption buffer and disrupted with a RiboLyser™ in four steps of 20 sec at the highest level. To remove cell fragments and glass beads, the extract was centrifuged at 14,000 rpm and 4 °C for 20 min. Afterward the supernatant was ultracentrifuged for 90 min at 45,000 rpm and 4 °C. The reaction assay was mixed in a photometer cuvette and contained the following substances in a total volume of 1 ml:

- 100 mM TES (pH 7.2)
- 0.9 mM TPP
- 3 mM cysteine
- 10 mM $MgCl_2$
- 2 mM NAD^+
- substrate
- 25 – 50 µl cell extract

The substrates pyruvate, 2-oxoglutarate, KMV, KIV or KIC were added in different concentrations. The test was performed at 30 °C and the reaction was started by the addition of 10 µl of a 16 mM solution of coenzyme A. The enzyme activity was followed by measuring the formation of NADH + H^+ at 340 nm wavelength with the photometer. The enzyme activity was calculated with the extinction coefficient of NADH $\varepsilon_{NADH340} = 6.22\ (mmol*cm)^{-1}$.

2-hydroxy acid dehydrogenase assay

A 2-hydroxy acid dehydrogenase is an enzyme that catalyzes the reduction of the branched-chain α-keto acids KMV, KIV and KIC to 2-hydroxy acids, whereupon the cosubstrate NADH is oxidized to NAD^+. To determine the activity of the 2-hydroxy acid dehydrogenase the method of Chambellon *et al*. (2009) was applied. Cells for the measurement of the 2-hydroxy acid dehydrogenase were grown in 2×TY-complex medium and in CgXII-minimal medium and harvested at an OD_{600} of 5. The following solutions are required for the preparation of cell extract:

Washing buffer:	50 mM triethanolamine (pH 7.3)
Disruption buffer:	100 mM KH_2PO_4/K_2HPO_4 (pH 7.3)
	0.5 mM DTT
	20 % glycerol

The cells were washed twice and stored at – 20 °C until further use. For the cell disruption with a RiboLyser™ the cell pellets were suspended in 1 ml disruption buffer. The reaction assay was mixed in a photometer cuvette and contained the following substances in a total volume of 1 ml:

- 100 mM KH_2PO_4/K_2HPO_4 (pH 7)
- 0.3 mM NADH + H^+
- cell extract

The mixture was incubated at 37 °C and the reaction was started by addition of the substrate pyruvate, KMV, KIV or KIC. Enzyme activity was detected with the photometer by measuring the oxidation of NADH + H^+ at 340 nm wavelength. The enzyme activity was calculated with the extinction coefficient of NADH $\varepsilon_{NADH340} = 6.22\ (mmol*cm)^{-1}$.

Homoserine dehydrogenase assay

The HOM catalyzes the reduction of aspartate semialdehyde to form homoserine, whereupon the reduction equivalent NADPH is oxidized to $NADP^+$. The HOM was assayed as described by Follettie *et al.* (1988). The following solutions are required for the preparation of cell extracts:

Washing buffer:	100 mM KH_2PO_4/K_2HPO_4 (pH 7.0)
	50 mM KCl

Disruption buffer: 100 mM KH_2PO_4/K_2HPO_4 (pH 7.0)

0.2 mM pyridoxal-5-phosphate

1 mM DTT

0.1 mM EDTA

Cells for the HOM measurement were grown in 2×TY-complex medium up to an OD_{600} of about 5, washed twice in washing buffer and stored at -20 °C until further use. For the preparation of cell extract the cell pellets were solved in disruption buffer and disrupted with a ribolyzer. The reaction mixture contained the following substances in a total volume of 1 ml:

- 100 mM KH_2PO_4/K_2HPO_4 (pH 7)
- 500 mM KCl
- 3 mM DL-aspartate-β-semialdehyde
- cell extract

The reaction was started by the addition of 0.4 mM NADPH + H^+ and the decrease of absorption by the oxidation of NADPH was followed at 340 nm wavelength at 30 °C in the photometer. The enzyme activity was calculated with the extinction coefficient of NADPH $\varepsilon_{NADPH340}$ = 6.22 $(mmol*cm)^{-1}$.

Threonine dehydratase assay

The threonine dehydratase catalyzes the desamination of threonine to form 2-oxobutyrate in the isoleucine biosynthesis pathway. Cell cultivation and preparation of cell extract for the measurement of the threonine dehydratase took place in the same way as described for the homoserine dehydrogenase assay. The reaction assay was mixed in an eppendorf reaction tube and contained the following substances (modified after Möckel *et al.*, 1992):

- 100 mM KH_2PO_4/K_2HPO_4 (pH 8.2)
- 1 mM pyridoxal-5-phosphate
- 40 / 80 mM L-threonine
- cell extract

For the inhibition studies up to 50 mM KMV, KIV or KIC were added to the test. The reaction started with the addition of the cell extract. Incubation took place at 30 °C. Since it was a discontinuous test, at four time points 200 µl of the assay mixture were taken and added to 1 ml detection reagent (1 g semicarbazide, 0.9 g sodium acetate / 100 ml), which stops the reaction. The detection mixture was incubated for at least 15 min at 30 °C, so that the semicarbazide

condensates with the reaction product 2-oxobutyrate to yield a semicarbazone (MacGee and Doudoroff, 1954), which could be detected photometrically at 254 nm wavelength. To calculate the 2-oxobutyrate concentrations a standard curve was made with several concentrations between 0.5 mM and 10 mM 2-oxobutyrate. The samples were diluted with water for the photometric detection. Since semicarbazide is not a specific detection reagent for 2-oxobutyrate, but also condensates with other ketones and aldehydes, among them KMV, KIV and KIC, the samples had to be diluted more heavily. For those tests a second standard curve was made, which was treated in the same way as the test samples.

AHAS assay

The AHAS catalyzes two reactions: Either the condensation of two molecules of pyruvate to form acetolactate in the valine synthesis or the analogous formation of acetohydroxybutyrate from pyruvate and 2-oxobutyrate with the isoleucine biosynthesis. The AHAS-assay was performed after the method of Leyval *et al.* (2003). The following solutions are required for the preparation of cell extract:

Washing buffer:	2 % [w/v] KCl solution
Disruption buffer:	100 mM KH_2PO_4/K_2HPO_4, pH 7.3
	0.5 mM DTT
	20 % glycerol

For the AHAS-measurement *C. glutamicum* was grown in CgXII-minimal medium with 2 % glucose and harvested at an OD_{600} of about 5. The cells were washed for three times. Between the washing steps the suspended cells were incubated on ice for 10 min. For the preparation of cell lysate, cell pellets were suspended in disruption buffer and disrupted using a RiboLyser™. The reaction mixture for the measurement of the pyruvate + pyruvate reaction of the AHAS contained the following substances in a total volume of 5 ml and was mixed in 15 ml falcon tubes.

- 100 mM KH_2PO_4/K_2HPO_4, pH 7.3
- 10 mM $MgCl_2$
- 0.1 mM FAD (Na salt)
- 0.1 mM TPP
- pyruvate
- 500 µl cell extract

For the determination of the K_m-value nine different pyruvate concentrations between 2.5 and 50 mM were applied to the test (between 5 and 60 mM if KMV was added as inhibiting substance). The reaction was started by addition of the cell extract and incubated at 37 °C with shaking. After 5, 10, 15 and 20 min samples of 1 ml each were taken and added to 100 µl 50 % H_2SO_4 to stop the reaction. The samples were incubated at 37 °C for 30 min whereupon the reaction product acetolactate is decarboxylated to acetoine. To evaluate the acetoine concentration 100 µl of the samples were mixed with 400 µl 0.45 N NaOH and 500 µl detection reagent (0.5 % creatine + 5 % α-naphtol in 2.5 N NaOH: v/v = 1/1) for 30 min at room temperature in the dark under shaking. The absorbance of the mixture was measured at 535 nm wavelength. The concentration was calculated with the help of an acetoine calibration curve for which samples containing 0.01 to 0.05 mM acetoine were treated in the same manner.

To measure the K_m-value of the 2-oxobutyrate + pyruvate reaction of the AHAS, the assay protocol was modified. The assay mixture contained 100 mM pyruvate as first substrate and nine different concentrations of 2-oxobutyrate between 1 and 40 mM as second substrate. The process of the test was the same, except that the samples were taken after 0, 30, 60 and 90 min. The product of this reaction, 2-aceto-2-hydroxybutyrate, could not be detected photometrically, so the enzyme activity was evaluated by the decrease of the substrate concentration. The concentration of 2-oxobutyrate was detected via HPLC using the derivatization method with DDB. Due to the very high pyruvate concentration in the samples, the DDB concentration had to be increased to 80 mg / 100 ml.

2.11 Chromatographic separation

HPLC analysis

Chromatographic separation techniques were used in this work to identify and quantify amino acids and α-keto acids in the culture supernatants of growth experiments and also to measure the 2-oxobutyrate + pyruvate reaction of the AHAS by measuring the decrease of the substrate. For this the method of a reversed phase (RP) high performance liquid chromatography (HPLC) was applied. The analytes which are in a polar mobile phase show hydrophobic interactions with a non polar stationary phase. By gradual reversal of the mobile phase into a non polar phase the

analytes are solved from the stationary phase and at this separated dependent on their hydrophobicity. Afterwards they are quantified with an appropriate detector.

Measurement of amino acids via HPLC

The quantification of amino acids via HPLC is based on a method from Lindroth and Mooper (1979). The amino acids are derivatized with ortho-phtaldialdehyde (OPA) directly before they are applied to the column to get fluorescing isoindole-complexes which can be measured with a fluorescence detector. The samples had to be diluted so that the concentrations were in the detection range of the method (5 – 100 µM). As an internal standard 100 µM ornithin was added to each sample. For the separation and detection of amino acid an Agilent LC 1100 HPLC with a fluorescent detector was used. The samples were taken and mixed with OPA with an automated program to ensure a derivatization under stable conditions (May and Brown, 1999). The separation over a pre column and a main column was performed at 40 °C with a flow rate between 0.75 ml/min. As mobile phase a mixture of the solutions A (100 mM sodium acetate, pH 7.2) and B (methanol) was used, starting with 80 % solution A. The concentration of methanol in the mobile phase was gradually increased in the course of the program. The detection with the fluorescence detector took place with an excitation wavelength of 230 nm and measured at an emission wavelength of 455 nm. To identify and quantify amino acids in the received chromatograms regression lines were made with known concentrations of the relevant amino acids. For this calibration and the analysis of the chromatograms the software HP-Chemstation for LC Rev. A.06.01 was used.

Measurement of α-keto acids via HPLC

α-keto acids were also transformed into fluorescing derivates and detected with a fluorescence detector. In contrast to the amino acids the derivatization could not be performed directly before the separation on the HPLC, but had to be done in a separate work step. For this the following derivatization solution was used:

derivatization reagent:	17 mg/50 ml 1,2-diamino-4,5-dimethoxybenzol (DDB)
	0.5 M HCl
	0.21 M β-mercapto ethanol

DDB is the actual fluorescent dye that forms derivates with α-keto acids in a selective reaction (Hara *et al.*, 1985). HCl serves as a catalyst for this reaction. β-mercapto ethanol stabilizes DDB so that the derivatization reagent can be stored in the dark at 4°C. The samples were diluted before the derivatization so that the α-keto acid concentrations are in the detection range between 5 and 100 µM. 100 µM oxovalerate was added as an internal standard. 200 µl of sample was mixed with 200 µl of the derivatization reagent and incubated for 2 hours at 105 °C in darkness. The reaction was stopped by a short incubation on ice. The separation and detection took place with an Agilent LC 1100 HPLC with a fluorescent detector. The separation over a pre column and a main column was performed at 40 °C with a flow rate between 0.35 and 0.5 ml/min. As mobile phase a mixture of solution A (H_2O) and solution B (methanol) was used, starting at a low methanol concentration which was increased in the course of the program. The detection with the fluorescence detector took place with an excitation wavelength of 361 nm and measured at an emission wavelength of 448 nm. To identify and quantify α-keto acids in the received chromatograms regression lines were made with known concentrations of the relevant α-keto acids. For this calibration and the analysis of the chromatograms the software HP-Chemstation for LC Rev. A.06.01 was used.

Alternative method for the determination of α-keto acids

For several α-keto acid measurements in fermentation supernatants an alternative method was applied with the use of an UV-detector. Double determinations with both methods showed that comparable results could be achieved. For this method no derivatization is necessary. The samples were diluted differently, since the detection range of this method lies between 1 and 30 mM. The separation was performed with an Agilent LC 1100 HPLC and pre and main columns for organic acids. As mobile phase 10 mM H_2SO_4 was used with a constant flow of 0.5 ml/min at 50 °C.

Gas chromatographic analysis

To find out if the KMV degradation product 2-methylbutyraldehyde was present in culture supernatants a gas chromatograph with a flame ionization detector was employed. 1 ml of the samples were mixed with 100 µl of an internal standard solution (110 mM isobutanol in 2 N HCl) in crimp top vials and closely sealed. The sample application was performed with a Hamilton syringe using an autosampler. The used column allows the separation of alcohols, aldehydes as well as organic acids.

The analysis was performed with the following chromatographic conditions:

Column: glas, packed (iØ 2mm × 2mm)

Column packaging: Chromosorb 101 (80-100 *mesh*)

Mobile phase: N_2 (33,5 ml/min)

Injector temperature: 195 °C

Detector temperature: 230 °C

Temperature profile: 130 °C for 1 min

Gradient from 130 °C to 200 °C with 4 °C/min

200 °C for 3 min

For data analysis the computer software "Maestro Sampler II" was applied. For the quantification calibration runs were made with calibration samples containing the analyzed substances in a concentration of 5 nm and 10 mM of the internal standard solution.

3. Results

3.1 Influence of KMV on *C. glutamicum*

3.1.1 Effect of KMV and other branched-chain α-keto acids on the growth of *C. glutamicum*

Most production strains have to cope with unnaturally high concentrations of the produced substance. Therefore the first step with the construction of such strains is to evaluate the overall behavior of the organism in the presence of this substance. Since the aim of the present work is the biotechnological production of KMV, *C. glutamicum* was grown in CgXII minimal medium with KMV and also with the other branched-chain α-keto acids KIV and KIC and growth was followed for 30 h. As shown in figure 4, *C. glutamicum* incubated without any branched-chain α-keto acids grew with a growth rate of 0.40 h^{-1} to a final OD_{600} of 33 with 2 % glucose as carbon source. All three branched-chain α-keto acids had a negative influence on the growth of *C. glutamicum*, the strongest inhibitor was KMV. A concentration of 50 mM KMV allowed growth with a rate of 0.15 h^{-1} up to an OD_{600} of around 5. With 50 mM KIV the growth rate was only slightly reduced from 0.40 h^{-1} to 0.27 h^{-1}. Medium with 50 mM KIC allowed growth with a rate of 0.15 h^{-1}, but in contrast to KMV an OD_{600} of 30 was reached.

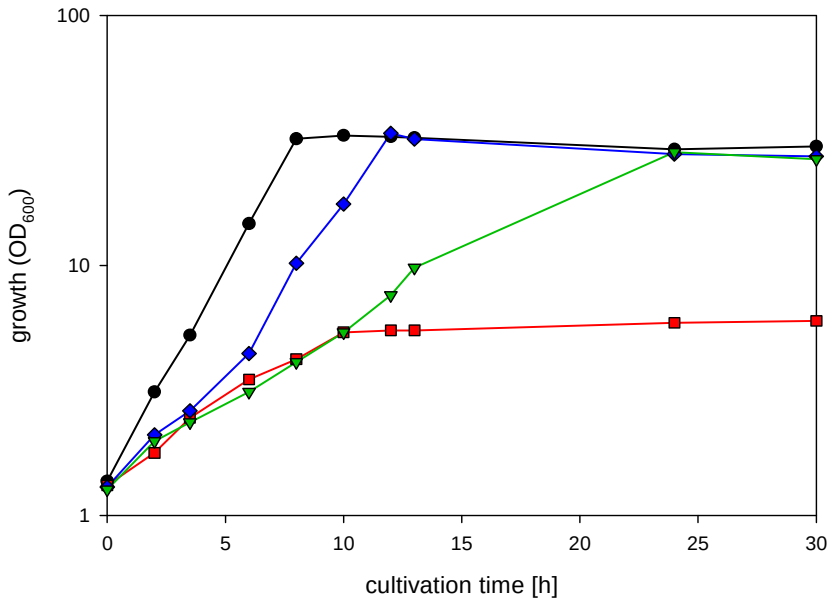

Figure 4: Growth of *C. glutamicum* WT in CgXII minimal medium + 2 % glucose. Addition of 50 mM KIV ◆, 50 mM KIC ▼, 50 mM KMV ■ or without further ingredients ●.

It was also tested, whether *C. glutamicum* is able to use KMV as carbon source. For this purpose *C. glutamicum* was cultivated in CgXII minimal medium without glucose as carbon source, but with 25 mM KMV. As shown in figure 5A, no growth occurred, and from this result it can be concluded that *C. glutamicum* is not able to use KMV as sole carbon and energy source. Addition of yeast extract to a culture containing glucose and 25 mM KMV allowed a much better growth than without complex ingredients. The culture started growing nearly as fast as a culture without KMV (growth rate 0.37 h^{-1}), but slowed down after five hours and only reached an OD_{600} of 23 while the reference culture reached an OD_{600} of 30. Yeast extract as a complex ingredient contains amino acids, among them L-valine, L-leucine and L-isoleucine which are synthesized from KIV, KIC and KMV by transaminase B.

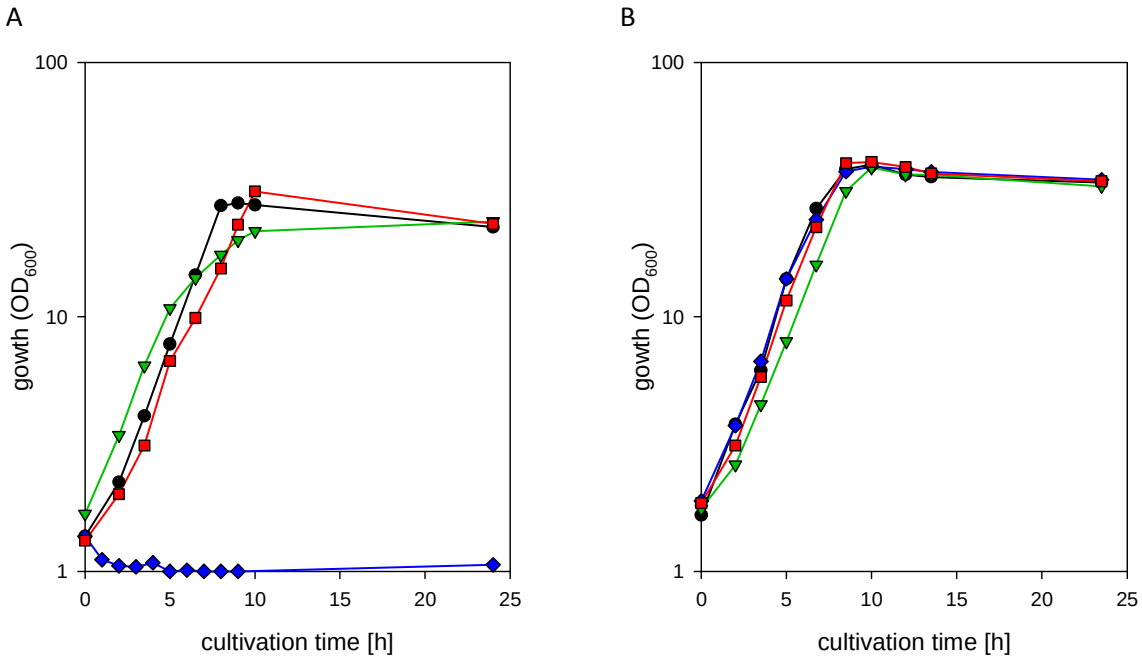

Figure 5: Growth of *C. glutamicum* in CgXII-minimal medium. **A:** with 2 % glucose ●, with 25 mM KMV◆, with 2 % glucose plus 25 mM KMV plus 0.5 % yeast extract ▼, with 2 % glucose plus 25 mM KMV plus L-valine, L-leucine and L-isoleucine (5 mM each) ■. **B:** Addition of 2 % glucose, 25 mM KIV plus L-leucine and L-isoleucine (5 mM each) ◆, 2 % glucose, 25 mM KIC plus L-valine and L-isoleucine (5 mM each) ▼, 2 % glucose, 25 mM KMV plus L-valine and L-leucine (5 mM each) ■, 2 % glucose ●.

Another growth experiment was performed with medium containing the three branch-chained amino acids together with 25 mM KMV. This resulted in a high growth rate of 0.38 h^{-1} which is on the same level as a culture without additional KMV (figure 5A). In contrast to the culture with yeast extract, the growth rate did not slow down in the later exponential growth phase and reached an OD_{600} of 30 as the reference culture did. Also addition of only valine and leucine to KMV led to a recovery of the growth rate (figure 5 B). This was also the case with KIV and KIC.

In further experiments the branched-chain α-keto acids were added to the cultures in combinations (figure 6). A combination of KMV and KIV only had the same inhibiting effect as KIV alone and did not show the strong growth inhibition of the KMV-containing culture. Thus, the addition of KIV could compensate the inhibiting effect of KMV. With KIC the same effect was observed. A culture that contains a combination of KIC and KMV (figure 6) reached the same growth rate as a culture with KIC alone (figure 4). And also a culture with all three branched-chain α-keto acids had a growth rate nearly as high as the KIV-culture. So the growth rate is dependent on the least inhibiting branched-chain α-keto acids, while the stronger effect of KMV could be compensated.

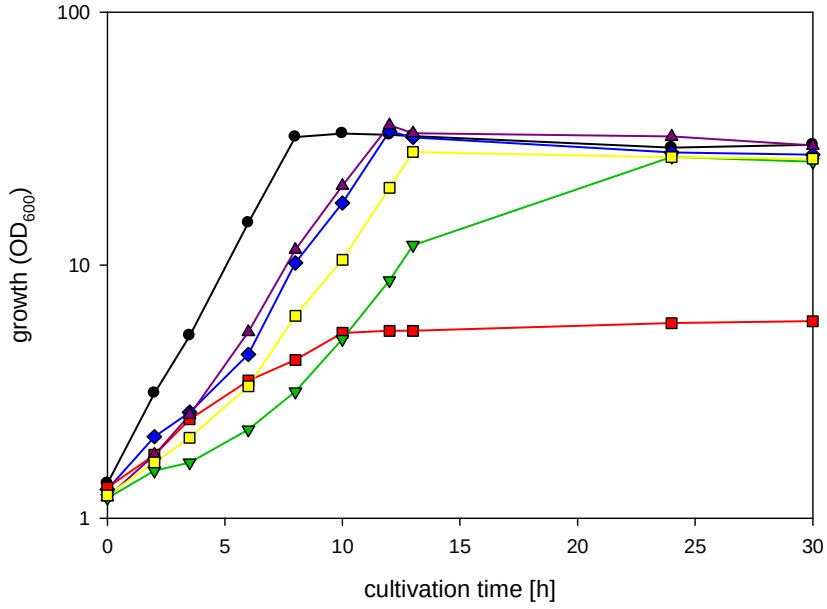

Figure 6: Growth of *C. glutamicum* WT in CgXII-minimal medium + 2 % glucose with branch-chained α-keto acids. Addition of 50 mM KIV ◆, 50 mM KMV ■, 50 mM KIV + KMV ▲, 50 mM KIC + KMV ▼, 50 mM KIV + KIC + KMV □ and without further additions ●.

3.2 Degradation of branched-chain α-keto acids

3.2.1 Degradation of branched-chain α-keto acids by *C. glutamicum* during growth

During the growth experiments with *C. glutamicum* together with the three branched-chain α-keto acids it became obvious, that their concentration decreased during growth and thus they were probably degraded. To analyze this more closely *C. glutamicum* again was cultured in the presence of KIV, KIC and KMV. In these experiments a lower branched-chain α-keto acid concentration of 25 mM was chosen so that all cultures were able to grow despite of the inhibiting effect. At several points of time samples were taken from the culture supernatant and the keto-acid concentration was measured via HPLC (figure 7). While the 25 mM KIV and KIC were almost degraded after 14 hours during the exponential growth phase, about half of the KMV was still present at the beginning of the stationary phase. Even after 48 hours 5 mM KMV remained. So again a difference was observed between the three branched-chain α-keto acids: While KIV and KIC were degraded very fast, the degradation of KMV was much slower.

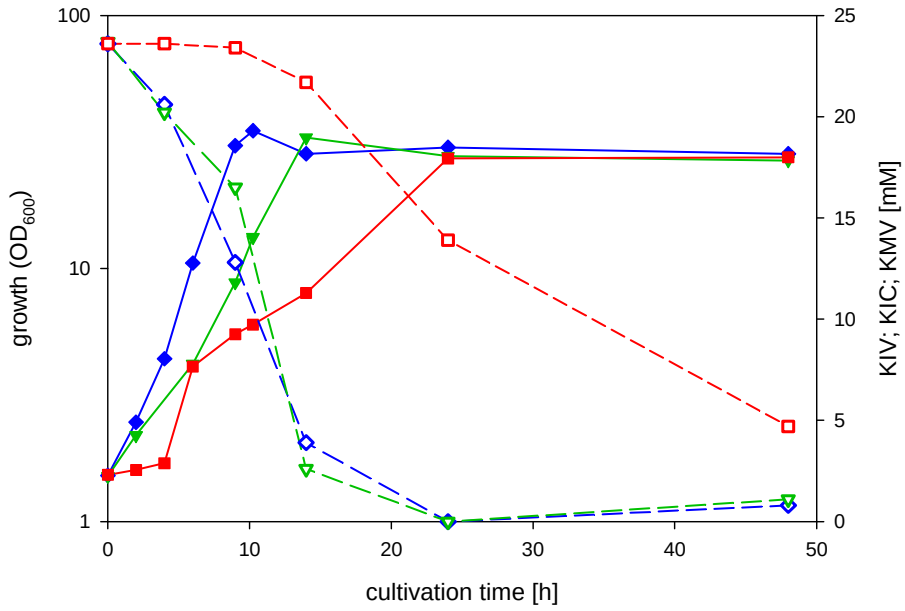

Figure 7: Growth of *C. glutamicum* WT in CgXII-minimal medium + 2 % glucose and branched-chain α-keto acids and measurement of the branched-chain α-keto acid concentration in culture supernatants. Growth with KIV ◆, KIC ▼ and KMV ■ . Concentration of KIV ◇, KIC ▽ and KMV □.

Next it was tested whether the degradation of branched-chain α-keto acids is inducible by their presence in the preculture. For this purpose *C. glutamicum* was cultured in CgXII-minimal medium with KIV, KIC and KMV (10 mM each) and with valine, leucine and isoleucine (10 mM each) and without additions. Additionally a 2×TY culture was made. All four cultures were used as precultures for another growth experiment in CgXII minimal medium with 2 % glucose and 25 mM KMV. All three cultures from a minimal medium preculture showed similar growth (figure 8 A), only the culture without additions grew slightly slower (growth rate 0.23 h^{-1}) than the other two cultures which had a growth rate of 0.28 h^{-1}. Consistent with the culture density, the KMV degradation starts a little bit slower in the culture which was not exposed to branched-chain α-keto acids or branched-chain amino-acids in the preculture (figure 8 B). The culture that was inoculated with a complex preculture grew significantly slower than the other three cultures (growth rate of 0.17 h^{-1}) and stopped growth at an OD_{600} of 6 after 8 hours. Also there was only a very low KMV degradation with this culture. The same experiment was also made with KIC and KIV. While with KIC the TY-culture grew slower than the cultures with minimal medium inoculants, all cultures had the same behavior when KIV was added to the medium (data not shown).

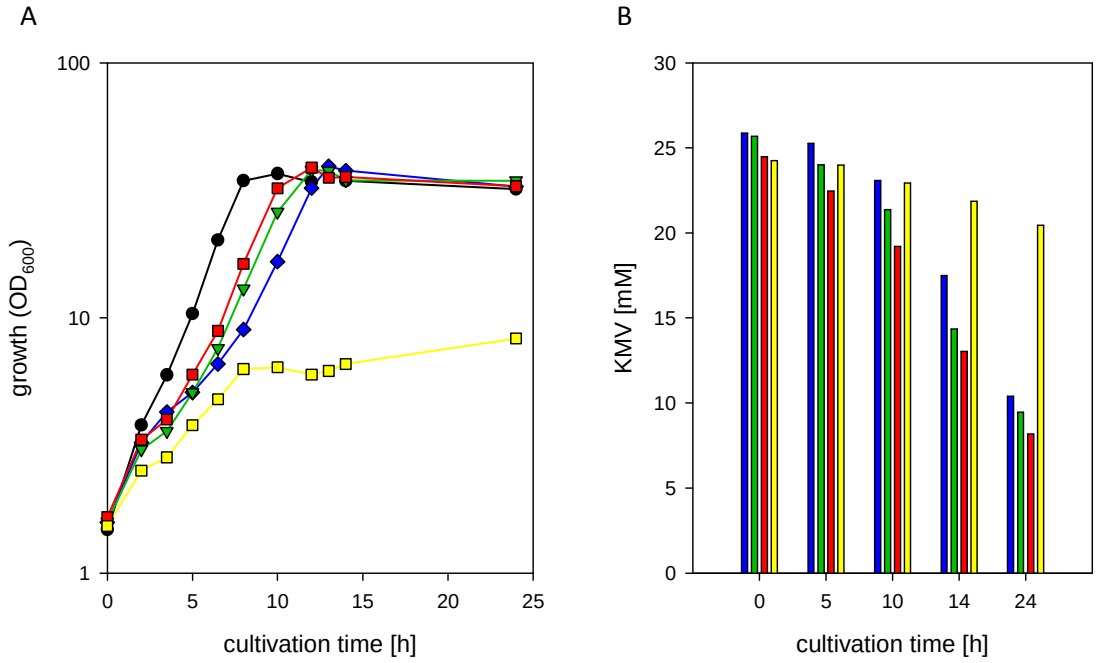

Figure 8: A: Growth of *C. glutamicum* in CgXII-minimal medium + 2 % glucose. Inoculants from different precultures: CgXII-minimal medium ◆ , CgXII + valine, leucine, isoleucine (10 mM each) ▼ , CgXII + KIV, KIC, KMV (10 mM each) ■ , 2×TY complex medium □ . All cultures except the reference culture (●) contained 25 mM KMV. **B:** KMV concentration in the culture supernatants from the experiments shown in A. Same color code as in A.

Further HPLC analysis of supernatants of cultures grown in the presence of KMV showed, that these cultures excreted isoleucine in an amount almost comparable to the degraded KMV (figure 9 A). In the presented experiment 13 mM isoleucine were excreted and the KMV concentration decreased by 18 mM. It is therefore most probable that the KMV is taken up by the bacteria, converted to L-isoleucine by the transaminase B and afterwards excreted into the medium. To verify this hypothesis, experiments were made with *C. glutamicum* Δ*ilvE*, which is transaminase B-negative, and therefore is auxotrophic for L-valine, L-leucine and L-isoleucine. This strain was also grown in the presence of the three branched-chain amino acids and the branched-chain α-keto acids (figure 9B). Already shown in the chapter before, the three amino acids compensated the inhibiting effect of the branched-chain α-keto acids, so there was no growth defect of the cultures in this experiment. The branched-chain α-keto acid degradation was significantly slower than with the wild type strain of C. glutamicum, but there still was degradation although no amino acids were excreted. After 48 hours of growth around 7 mM KIV, 11 mM KIC and 14 mM KMV were left from 25 mM branched-chain α-keto acids present at the beginning of the

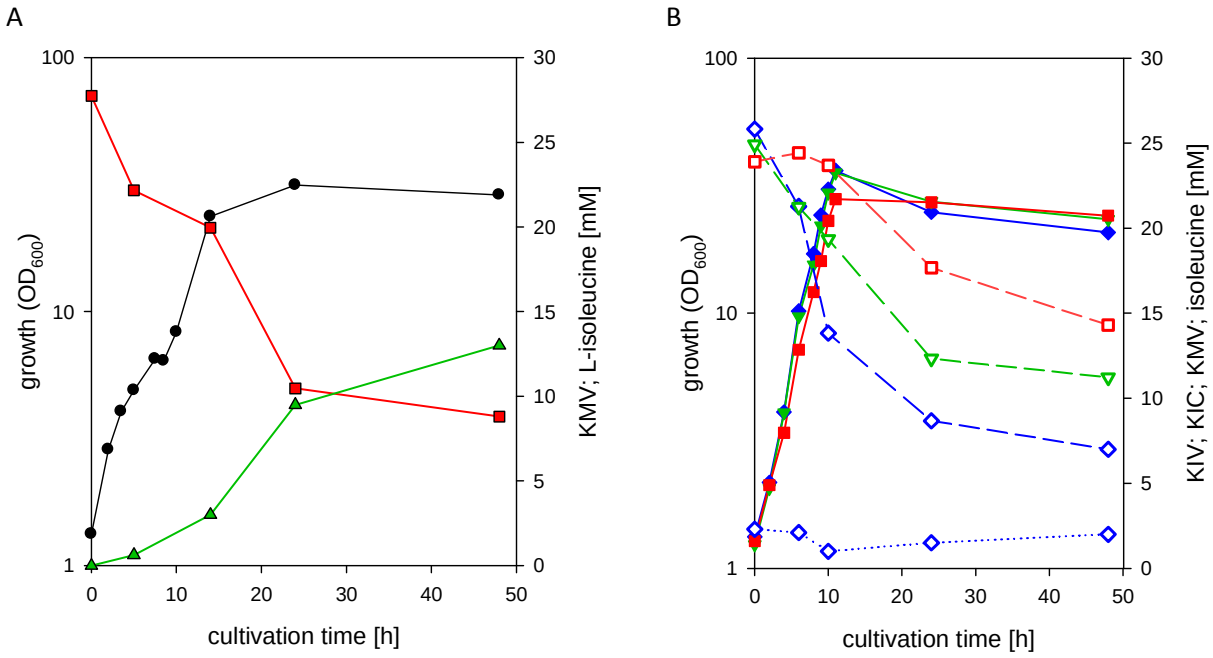

Figure 9: A: Transformation of KMV into L-isoleucine by *C. glutamicum*. Growth in CgXII-minimal medium + 2 % glucose + 25 mM KMV. The graph shows growth ● , KMV-concentration ■ and L-isoleucine concentration ▲ in the supernatant. **B:** Keto acid degradation with *C. glutamicum* Δ*ilvE*. Growth in CgXII-minimal medium + 2 % glucose + L-valine, L-leucine, L-isoleucine (2 mM each) + 25 mM KIV (blue diamond), 25 mM KIC (green triangle) or 25 mM KMV (red square). Filled symbols show the growth, open symbols the keto acid concentration in the supernatant. The dotted curve shows the isoleucine concentration in the KMV-culture.

experiment. The isoleucine concentration in the KMV-culture was also measured, but no excretion could be detected. The measured amounts represent the isoleucine that was supplemented to the medium. Hence there must be another mechanism for the degradation of branched-chain α-keto acids next to the conversion into the corresponding amino acids.

3.2.2 Stability of branched-chain α-keto acids in the presence of corn steep liquor

To test whether branched-chain α-keto acids are chemically stable under the conditions employed for the described experiments, they were incubated in cell-free medium. In a second approach corn steep liquor (CSL) was used as an additional medium component. CSL is a complex medium component which is widely used in the industrial biotechnological production. CSL is a byproduct of industrial corn wet milling, when starch is obtained by incubating corn in water. It contains the water soluble substances of the corn, such as sugars and organic acids as well as amino acids and several trace elements necessary for the growth of bacteria (Ligget and Koffler 1948; Frères and Lestrem, 2001). It was evaluated whether CSL has a beneficial effect on the stability of branched-chain α-keto acids during fermentation with *C. glutamicum*. Firstly the stability of KIV, KIC and KMV in cell-free medium at 30 °C with and without CSL was tested. In medium without CSL only KIV showed a slight tendency for degradation (figure 10 A). The

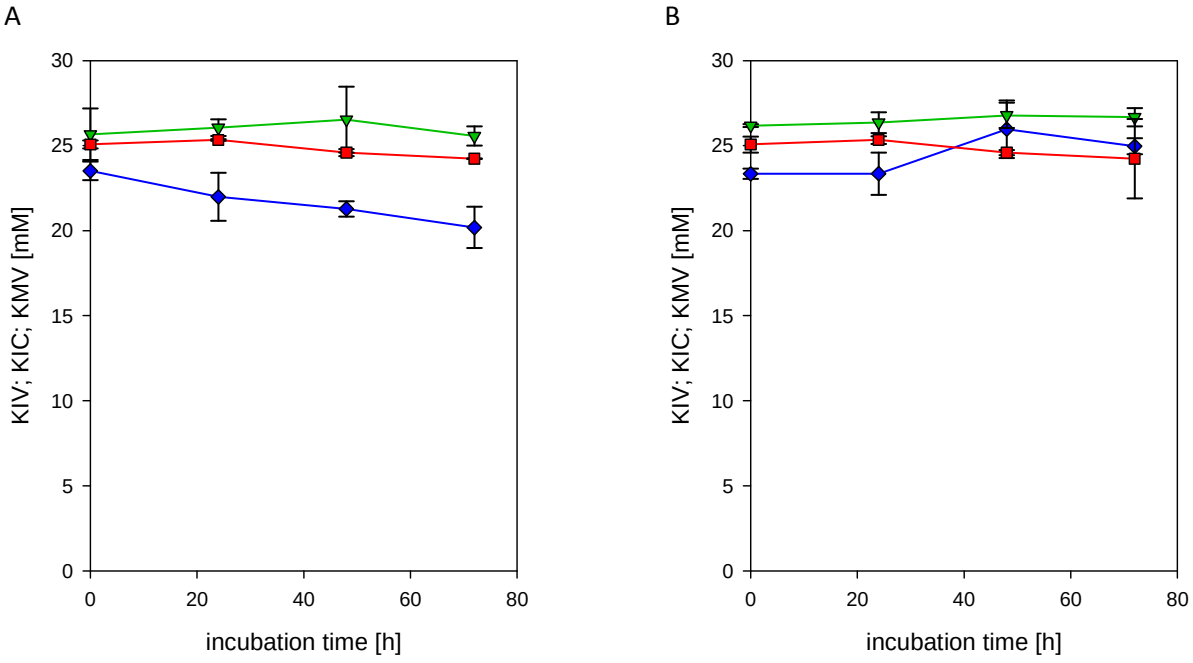

Figure 10: Stability of KIV ◆, KIC ▼ and KMV ■ during incubation in cell-free CgXII-minimal medium + 2 % glucose without (**A**) or with (**B**) CSL.

concentration of KIV was 10 to 15 % lower after three days of incubation. KIC and KMV were completely stable during this period of time. In the medium with CSL there was no detectable degradation with all three branched-chain α-keto acids (figure 10 B).

In a further approach the effect of CSL on growing cells and their branched-chain α-keto acid degradation rate was determined. Therefore *C. glutamicum* Δ*ilvE* was cultivated in CgXII-minimal medium with and without CSL and the addition of KIV, KIC or KMV. Again only with KIV a difference became visible when the cultures were compared (Fig 11 A). With KIC and KMV no different behavior appeared in the degradation of the branched-chain α-keto acids (Fig 11 B, C). Especially during the first ten hours of the growth the degradation of KIV was considerably faster in the culture without CSL. This led to a difference in the KIV-concentration of around 5 mM at the end of the experiment. Another remarkable aspect of the KIV degradation was the excretion of KIC during the exponential growth phase. The maximum KIC concentration after ten hours was higher in the culture without CSL (10 mM) than in the CSL supplemented culture, where 4 mM KIC were measured. The most probable explanation for this effect is that KIV is transformed into KIC by the bacteria.

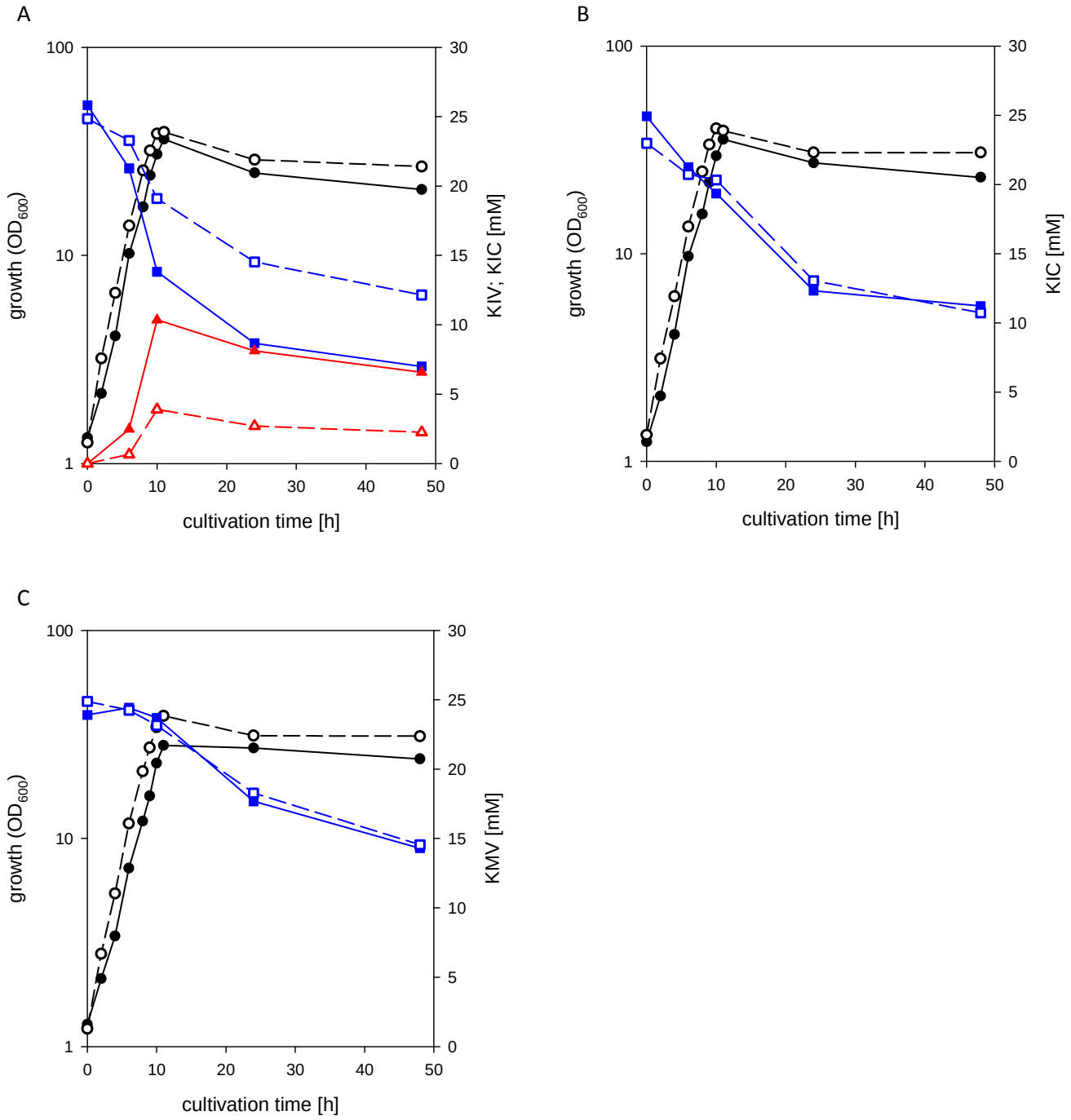

Figure 11: Effect of the complex medium component CSL on the degradation of KIV (**A**), KIC (**B**) and KMV (**C**). Growth of *C. glutamicum* Δ*ilvE* in CgXII-minimal medium + 2 % glucose + L-valine, L-leucine and L-isoleucine (2 mM each) with (open symbols) or without (filled symbols) addition of 1 % CSL. Documentation of the growth ●, the respective keto acid concentration ■ and the KIC concentration ▲ (only 8 A).

3.2.3 Enzymatic degradation of branched-chain α-keto acids

As shown in the previous chapters, *C. glutamicum* is able to degrade branched-chain α-keto acids. *C. glutamicum* Δ*ilvE* also degrades the branched-chain α-keto acids, although it is not able to transform them into the corresponding amino acids. Therefore, there has to be another pathway or enzyme for the degradation of KMV, KIV and KIC. One such enzyme might be the branched-chain α-keto acid dehydrogenase complex (BKDHC), which has been described for *Bacillus subtilis* and *Enterococcus faecalis* (Wang et al., 1993; Ward et al., 2000). This is an enzyme complex consisting of three subunits similar to the PDHC and the ODHC. Same as these enzyme complexes the BKDHC also would need coenzyme A as a cofactor. To test whether such an enzyme complex is present in *C. glutamicum*, *C. glutamicum* Δ*ilvA* Δ*panBC* was used, which is not able to synthesize pantothenate. Pantothenate is a precursor for the synthesis of coenzyme A, so a BKDHC should no longer be able to work in this strain. *C. glutamicum* Δ*ilvA* Δ*panBC* was cultured in 2×TY complex medium and afterwards in minimal medium to starve the bacteria out of pantothenate. In the main experiment, cultures with and without supplementation of pantothenate were compared in their ability to degrade KIV, KIC and KMV. As a reference the same experiment was performed with the wild type strain of *C. glutamicum*. When *C. glutamicum* Δ*ilvA* Δ*panBC* was supplemented with pantothenate, there was no difference in growth compared to the wild type with a growth rate of 0.36 h^{-1} and a maximal OD_{600} of around 30 (figure 12). There also was no significant difference with the degradation of branched-chain α-keto acids when grown in the presence of pantothenate (table 1). When starved out of pantothenate however, *C. glutamicum* Δ*ilvA* Δ*panBC* showed a very restricted growth. Under these conditions, *C. glutamicum* Δ*ilvA* Δ*panBC* degraded the branched-chain α-keto acids much slower than before. But if the degraded amount of branched-chain α-keto acids is put into relation with the OD_{600} as shown in table 1, the degradation is even higher for KIV and KIC. Only for KMV not only the absolute amount is lower in the starved out strain, but also the degradation rate. The strong effect the pantothenate deprivation had on the growth makes the branched-chain α-keto acid degradation of the pantothenate-limited cultures difficult to compare with the wild type strain. The existence of a BKDHC could neither be affirmed nor excluded by the current experiment.

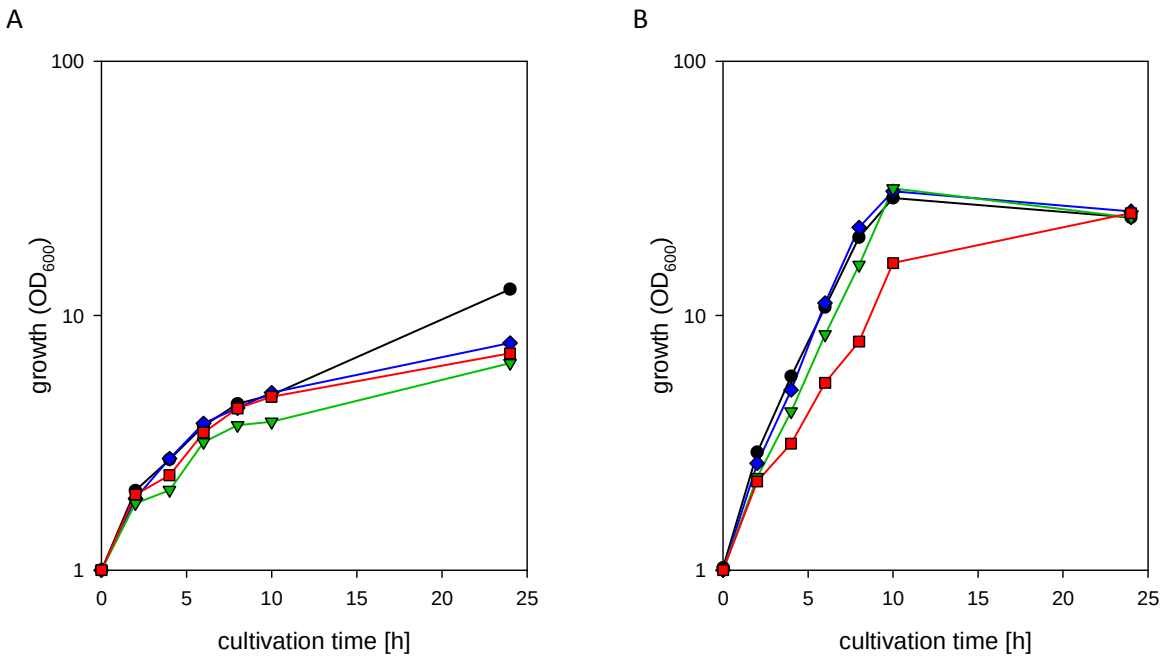

Figure 12: Growth of *C. glutamicum* Δ*ilvA* Δ*panBC* in CgXII-minimal medium + 2 % glucose + 2 mM L-isoleucine (compensation of the *ilvA*-deletion). Addition of 15 mM KIV ◆, 15 mM KIC ▼, 15 mM KMV ■ or without additions ●. **A:** without pantothenate **B:** with 0.001 mM pantothenate.

Table 1: Degradation of branched-chain α-keto acids by *C. glutamicum* WT and *C. glutamicum* Δ*ilvA* Δ*panBC* with and without supplementation of pantothenate.

strain	time [h]	concentration [mM]			degradation / OD_{600} [mM]		
		KIV	KIC	KMV	KIV	KIC	KMV
C. glutamicum WT without pantothenate	0	15.7	15.6	14.5			
	8	8.5	4.2	14.0	0.4	0.9	0.1
	24	0.0	0.0	3.9	0.6	0.6	0.4
C. glutamicum WT with pantothenate	0	17.2	16.0	14.0			
	8	7.5	4.9	12.6	0.5	1.0	0.1
	24	0.0	0.0	4.3	0.7	0.7	0.4
C. glutamicum Δ*ilvA* Δ*panBC* without pantothenate	0	16.2	15.4	13.3			
	8	11.6	10.0	13.2	1.1	1.5	0.0
	24	11.4	3.7	11.6	0.7	1.8	0.2
C. glutamicum Δ*ilvA* Δ*panBC* with pantothenate	0	14.4	15.9	14.9			
	8	7.5	2.3	11.6	0.3	0.9	0.5
	24	0.0	0.0	3.5	0.6	0.7	0.5

In another approach to find out if there is a BKDHC in *C. glutamicum* or if the PDHC or ODHC unspecifically catalyze the degradation of branched-chain α-keto acids the strains *C. glutamicum* ins*ldp* and *C. glutamicum* Δ*odhA* were employed. *lpd* codes for the enzyme 3 (dihydrolipoamide dehydrogenase) both of the PDHC and the ODHC. Since a lipoamide dehydrogenase is also necessary for a branched-chain α-keto acid dehydrogenase reaction, the PDHC or ODHC dihydrolipoamide dehydrogenase could also be a part of this complex. *odhA* codes for the enzyme I of the ODHC and is specific for this complex. The *odhA*-deletion mutant was chosen to evaluate, whether the branched-chain α-keto acid degradation is a side reaction of the ODHC.

C. glutamicum ins*ldp* grew very similar to the wild type in the presence of the branched-chain α-keto acids. As with the wild type KMV had the strongest inhibiting effect, more than KIC and KIV, the latter did not affect the growth rate at all (figure 13 A). The same became visible with the

branched-chain α-keto acid degradation where there were no significant differences compared to the wild type strain of *C. glutamicum*. 25 mM KIV and KIC were almost completely degraded after 14 hours, while 10 mM KMV were still present after 24 hours.

C. glutamicum Δ*odhA* turned out to possess a very problematic growth (figure 13 B). The strain had a long lag phase before starting exponential growth. Therefore it is difficult to compare it to the wild type. With this strain only KIC had an impact on the growth. While the KIV- and KMV-containing cultures grew very similar to the reference culture without addition of branched-chain α-keto acids, the culture with KIC took about ten hours longer to reach the final OD_{600}. Degradation of branched-chain α-keto acids already started during the 30 hour long lag phase and accelerated in the exponential growth phase. When reaching the stationary phase *C. glutamicum* Δ*odhA* had degraded most of the KIV and KIC while again around 10 mM KMV remained in the stationary growth phase.

The described experiments with *C. glutamicum* ins*ldp* and *C. glutamicum* Δ*odhA* indicated that neither the PDHC nor the ODHC are responsible for the degradation of branched-chain α-keto acids by unspecific reactions, since a concentration decrease was also observed with these strains.

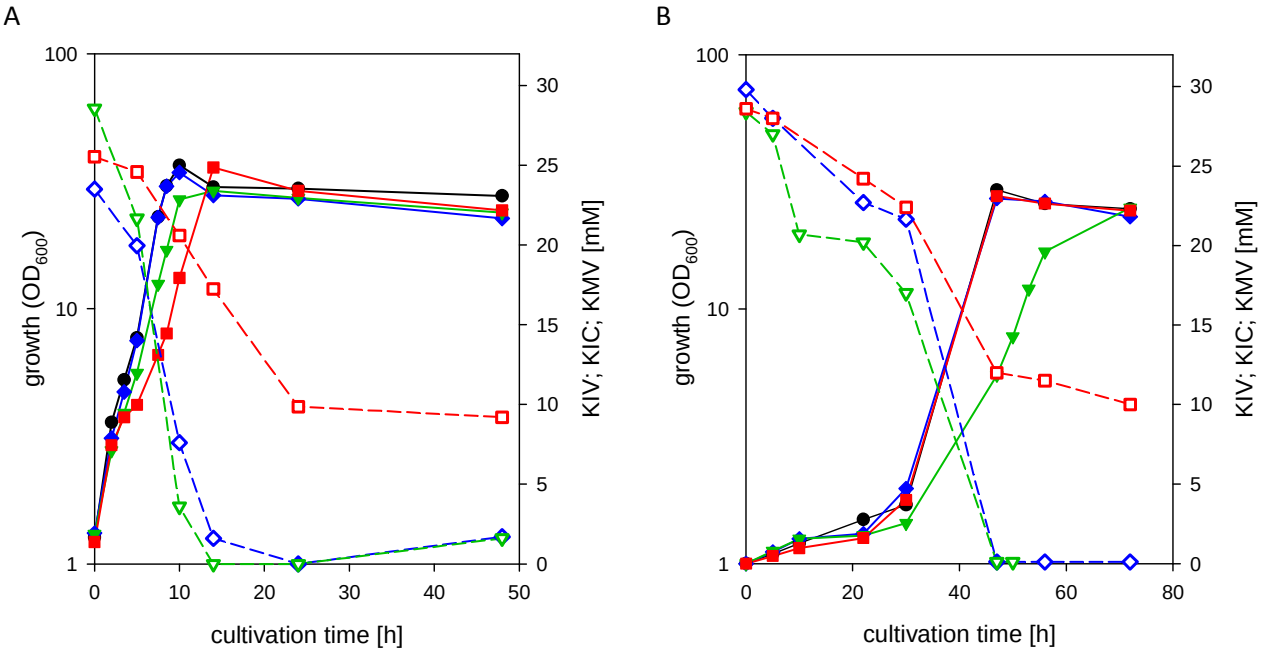

Figure 13: Growth of *C. glutamicum* ins*ldp* (**A**) and *C. glutamicum* Δ*odhA* (**B**) in CgXII-minimal medium + 2 % glucose with the addition of 25 mM KIV ◆, KIC ▼, KMV ■ or without further additions ●. The filled symbols show the growth, open symbols the branched-chain α-keto acid concentrations in the supernatant.

Table 2: Enzyme assay for the measurement of the PDHC, the ODHC and a possible BKDHC in *C. glutamicum*.

substrate	specific enzyme activity [µmol/min*mg protein]	
	2×TY complex medium	CgXII minimal medium
pyruvate (3 mM)	45.9 ± 5.3	26.9 ± 14.2
2-oxoglutarate (1.5 mM)	20.4 ± 5.2	10.3 ± 3.1
KIV (5-50 mM)	< 0.1	< 0.1
KIC (5-50 mM)	< 0.1	< 0.1
KMV (5-50 mM)	< 0.1	< 0.1

The existence of a BKDHC can still be possible in *C. glutamicum*, if the enzyme complex does not share the dihydrolipoamide dehydrogenase of the PDHC and the ODHC. To test this hypothesis, an enzyme assay was established to detect the activity of branched-chain α-keto acid degradation. As positive controls, the activities of the PDHC and the ODHC were determined, since they catalyze their reactions under the same conditions (table 2) as a proposed BKDHC. The activities were both measured in cell extracts from bacteria cultured in complex and in minimal medium. The PDHC and ODHC activities were about twice as high in cells grown in complex medium than in the cells grown in minimal medium. With both extracts, no activity could be measured when KIV, KIC or KMV were used as substrate for the enzyme assay. The branched-chain α-keto acids were used in high concentrations (up to 50 mM) to detect a BKDHC activity even if the enzyme has a very low affinity to its substrate. However, even under these conditions no activity was detectable. To exclude the possibility, that the BKDHC has to be induced by its substrates, cells were grown in the presence of KIV, KIC and KMV. However, even if all three branched-chain α-keto acids were added to the medium, no activity was detectable in the respective cell extract. Thus, it is very unlikely that *C. glutamicum* possesses a BKDHC.

Table 3: Specific 2-hydroxy acid dehydrogenase activity in *C. glutamicum*.

Substrate	specific activity [µmol/min*mg protein]
pyruvate	0.17 ± 0.02
KIV	< 0.1
KIC	< 0.1
KMV	< 0.1

A different enzyme that degrades branched-chain α-keto acids is the D-2-hydroxy acid dehydrogenase, as it has been described in *Lactococcus lactis* (Chambellon et al., 2009). This enzyme reduces the KIV, KIC and KMV to their respective 2-hydroxy acids while oxidizing NADH+H+ to NAD+. In *L. lactis* the probable role of this reaction is the regeneration of NAD+ when catabolizing branched-chain amino acids. To test for a 2-hydroxy acid dehydrogenase activity, cells of *C. glutamicum* WT were grown in minimal medium both in the presence and absence of KIV, KIC and KMV. In none of the tested cell extracts any enzyme activity was measured when KIV, KIC or KMV were used as substrates (table 3). NADPH+H+ was also used as a cosubstrate instead of NADH+H+, but again no activity was detectable. Pyruvate was used as a positive control for the test. With this substrate a specific activity of 0.17 U/mg protein was detected, which could be the activity of the lactate dehydrogenase, since this enzyme performs the analog reduction reaction with pyruvate. Thus, as with the BKDHC the existence of a 2-hydroxy acid dehydrogenase in *C. glutamicum* is very improbable.

3.2.4 Incubation of the branched-chain α-keto acids in "spent" medium

During the experiments with KIV, KIC and KMV as medium additions there were hints that the branched-chain α-keto acids are not completely stable in cell-free culture supernatants. Therefore experiments were made with medium of growing cultures of *C. glutamicum* WT and also medium of cultures in the stationary growth phase which was sterilized via filtration. KIV, KIC and KMV were incubated at 30 °C with shaking in such "spent" media and the concentration was measured via HPLC. It became obvious that in contrast to fresh medium the branched-chain α-keto acids were not stable, but there was quite a severe degradation, especially with KIV (figure 14 A). Here a concentration decrease of 10 to 15 mM in three days was detected. KIC and KMV were more stable also in the "spent" medium with a concentration decrease of about 5 mM. The degradation was significantly stronger in the medium of a growing culture which was harvested 8 hours after inoculation than in the supernatant of stationary cultures. Thus it is

unlikely that the degradation is caused by intracellular proteins which are set free into the medium by cell lysis. To elucidate if the degradation is caused by an enzyme catalyzed reaction, the medium was heated to 90 °C for 15 min so that contained proteins are denaturized. The stability of the branched-chain α-keto acids in this heated medium was compared to medium that was not treated in this way. As shown in figure 14 B the heating of the medium did not reduce the degradation of KIV, KIC and KMV and thus it is most probably not caused by the activity of excreted proteins. The same experiments were also performed with supernatants of cultures of the KMV production strain *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) whose construction is described in the following chapters. However, no differences were observed in comparison to the supernatants of *C. glutamicum* WT (data not shown).

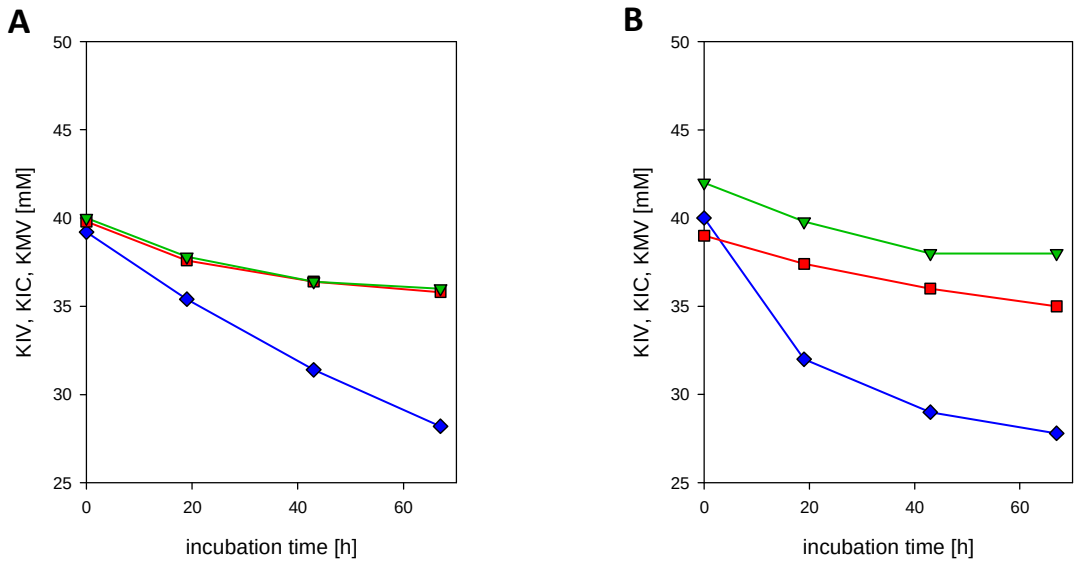

Figure 14: Incubation of KMV ■, KIC ▼and KIV ◆ in "spent" medium of a *C. glutamicum* WT culture (**A**). Incubation in "spent" medium that was heated at 90 °C for 15 min (**B**).

3.3 Characterization of key enzymes of the KMV biosynthesis

The AHAS and the TD are regarded as key enzymes of the KMV biosynthesis and are likely to be influenced in their activity by the branched-chain α-keto acids. Both enzymes are regulated by branched-chain amino acids and the AHAS uses the α-keto acids pyruvate and 2-oxobutyrate as substrates, so a impact of high concentrations of KIV, KIC or KMV on the enzyme activity could occur due to the structural similarities. Thus, enzyme assays for the AHAS and the TD were established to analyze those assumptions.

3.3.1 Characterization of the acetohydroxy acid synthase

The AHAS underlies a complex regulation, since it is both part of the isoleucine and the valine biosynthesis. The pyruvate + pyruvate reaction is known to be inhibited by the three branched-chain amino acids L-isoleucine, L-valine and L-leucine (Eggeling *et al.*, 1998; Elisáková *et al.*, 2005; Blombach *et al.*, 2009). To analyze if also the structurally related molecule KMV inhibits the AHAS, enzyme assays were made in the presence of KMV and the maximal specific enzyme activity as well as the K_m-value were evaluated. The Michaelis-Menten curve of the pyruvate + pyruvate reaction of the AHAS with 100 mM KMV is shown in comparison to the curve without KMV (figure 15 A). As shown in figure 15 A, the affinity of the enzyme towards pyruvate decreased, when 100 mM KMV was added to the enzyme assays. For the calculation of the K_m-value and the V_{max}, the Hanes-Wilkinson equation was used (figure 15 B). Here a parallel shift of the equation becomes obvious, which is typical for an increased K_m-value. The K_m-values calculated with the Hanes-Wilkinson equation increased about three-fold from 8.5 mM to 23 mM pyruvate in the presence of KMV (table 4). The maximal enzyme activity almost remained on the same level, with 90 mU / mg of protein without and 85 mU / mg of protein with 100 mM KMV. Because the 2-oxobutarate + pyruvate reaction is more important for the synthesis of KMV, it was tested whether this reaction is also affected by KMV in the same way. The measured specific activities for the 2-oxobutyrate + pyruvate reaction were higher than with the pyruvate + pyruvate reaction (figure 15 C). Here a maximal activity of about 130 mU / mg of protein was calculated (table 4). The K_m-value for 2-oxobutyrate (6.5 mM) was slightly lower than for pyruvate (8.5 mM). The addition of 100 mM KMV hardly had an effect on the 2-oxobutyrate + pyruvate reaction of the AHAS. The V_{max} did not change and the K_m-value only slightly increased to 7 mM 2-oxobutyrate. In summary the observed characteristics of the AHAS are beneficial for the biosynthesis of KMV, since 2-oxobutyrate is the preferred substrate of the enzyme and KMV did not negatively influence the reaction relevant for KMV-synthesis.

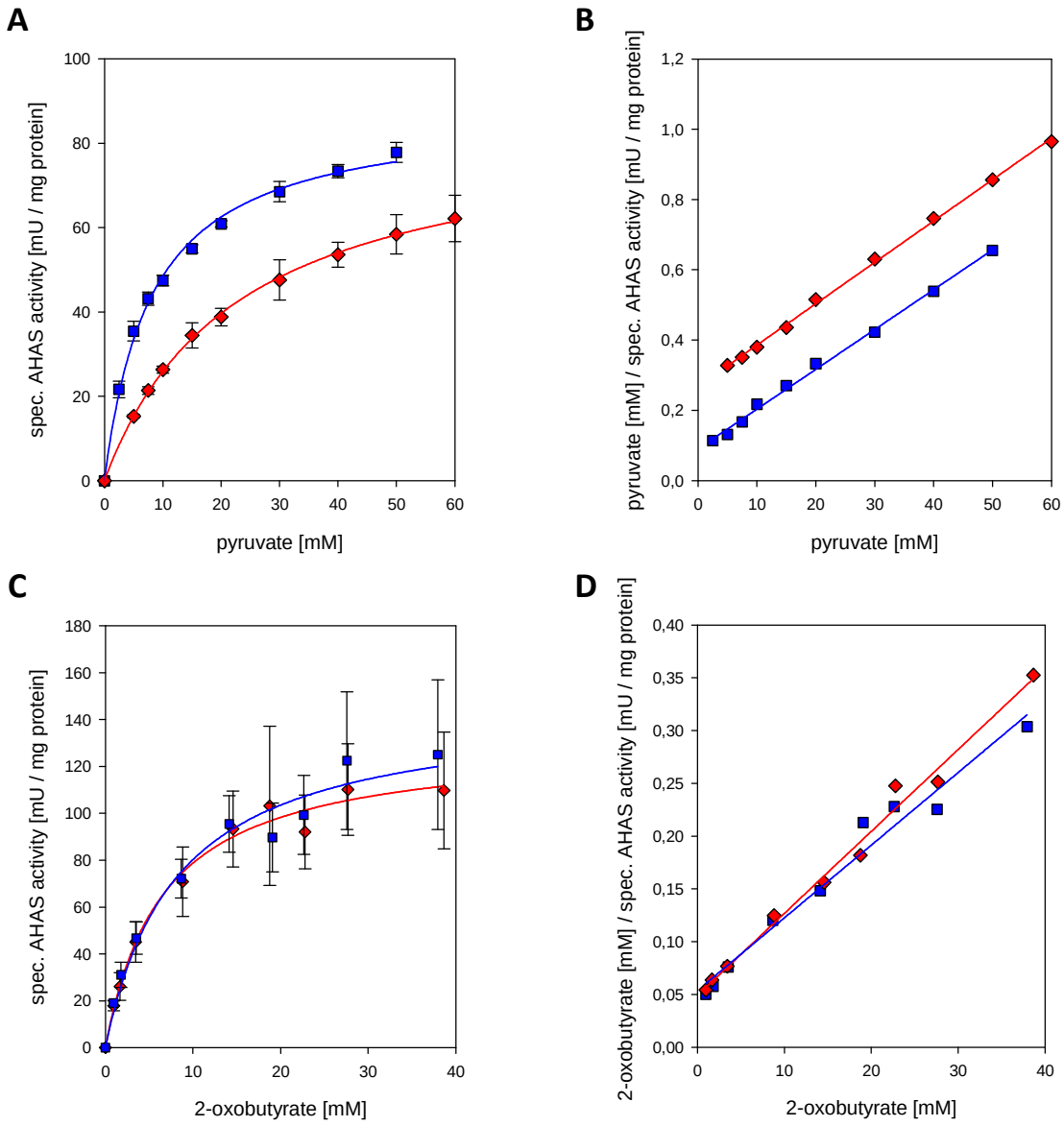

Figure 15: Determination of K_m-value and V_{max} of the AHAS. Pyruvate + pyruvate reaction (**A + B**), pyruvate + 2-oxobutyrate reaction (**C + D**). **A** and **C** show Michaelis Menthen diagrams, **B** and **D** the Hanes-Wilkinson equations of the AHAS-assays. Enzyme assays without addition ■, addition of 100 mM KMV◆.

Table 4: K_m-values and maximal specific activities (calculated with Hanes-Wilkinson equations) of the pyruvate + pyruvate and the 2-oxobutyrate + pyruvate reaction of the AHAS in the presence of different inhibiting substances.

	pyruvate + pyruvate		pyruvate + 2 -oxobutyrate	
	K_m-value for pyruvate [mM]	V_{max} [mU / mg protein]	K_m-value for 2-oxobutyrate [mM]	V_{max} [mU / mg protein]
without additions	8.5 ± 1.1	89.4 ± 3.7	6.4 ± 1.1	132.8 ± 6.0
+ 100 mM KMV	22.7 ± 2.1	85.0 ± 9.3	7.0 ± 0.6	132.9 ± 21.4
+ 100 mM KIV	n. t.	n. t.	6.2 ± 2.6	80.0 ± 7.8
+ 10 mM L-isoleucine	n. t.	n. t.	3.8 ± 1.5	90.3 ± 17.1
+ 10 mM L-valine	n. t.	n. t.	2.3 ± 0.0	79.5 ± 2.1

n. t. = not tested

KIV has been shown to be a stronger inhibiting agent than KMV with the pyruvate + pyruvate reaction of the AHAS (Krause *et al.*, 2010). Here it was tested if this is also the case for the 2-oxobutyrate + pyruvate reaction and thus, the AHAS activity was also measured in the presence of 100 mM KIV. As with the addition of KMV the K_m-value was not affected by KIV. For the 2-oxobutyrate concentration sufficient for half-maximal activity a value of 6.2 mM was calculated (table 4). In contrast to this the V_{max} decreased by about 40 % to a specific activity of 80 mU / mg of protein.

The pyruvate + pyruvate reaction of the AHAS is known to be repressed by the three branched-chain amino acids valine, leucine and isoleucine, but the 2-oxobutyrate + pyruvate reaction was not yet examined on this aspect. In this work the K_m-values and maximal enzyme activities of the AHAS in the presence of 10 mM L-valine and L-isoleucine were also measured for the 2-oxobutyrate + pyruvate reaction. The calculated maximal activities were reduced by 30 to 40 % similar to the addition of KIV (table 4). Remarkable are the lower K_m-values for 2-oxobutyrate when L-isoleucine or L-valine was added, which were calculated to be 3.8 mM and 2.3 mM 2-oxobutyrate, respectively.

3.3.2 Inhibition assays with the biosynthetic threonine dehydratase and branched-chain α-keto acids

The TD catalyzes the desamination of L-threonine to form 2-oxobutyrate, which is the first step of isoleucine and KMV synthesis. The enzyme is known to be strictly regulated by L-isoleucine. KMV is structurally similar both to the product 2-oxobutyrate and to L-isoleucine. Therefore it was self-evident to evaluate whether KMV has an inhibiting effect on the activity of the TD. Cell extracts of *C. glutamicum* (pMM36ptac *ilvA*) were used for these experiments. The tests were performed with a substrate concentration of 40 mM and 80 mM L-threonine. Increasing concentrations up to 50 mM KMV were added to the test. For comparison 1 mM L-isoleucine was used as inhibiting substance. With 40 mM L-threonine as substrate, the specific enzyme activity decreased with higher KMV concentrations (figure 16). With 50 mM KMV the activity was 50 % lower than without KMV (2.95 U / mg protein and 5.66 U / mg protein, respectively). 1 mM L-isoleucine led to an inhibition of more than 80 %. With 80 mM L-threonine as substrate the specific TD activity without KMV increased to 7.2 U / mg protein. With this substrate concentration the KMV effect on enzyme activity was much smaller. An inhibition of less than 15 % was observed when 10 mM KMV were added to the test. Higher KMV-concentrations did not have any further impact on TD activity.

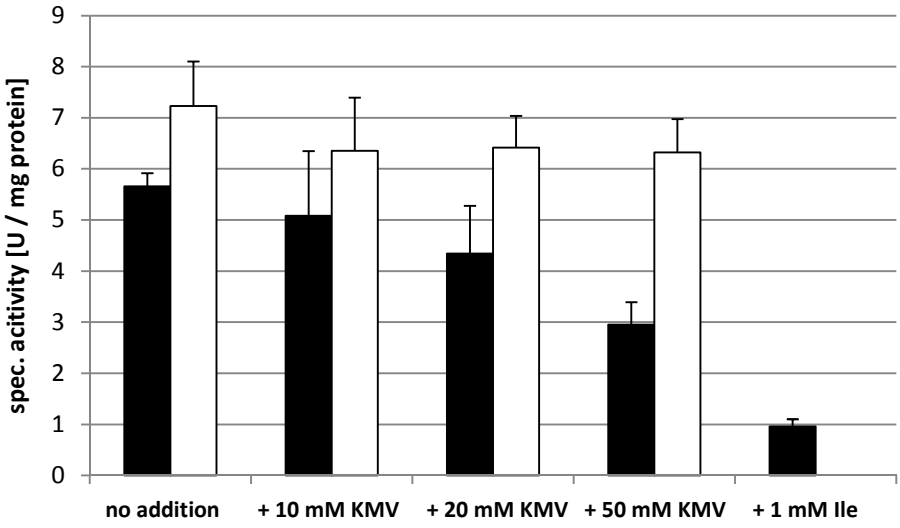

Figure 16: Specific activity of the TD with 40 mM (black) or 80 mM (white) L-threonine as substrate. Addition of different concentrations of KMV or 1 mM L-isoleucine.

In contrast to the strong inhibiting effect of L-isoleucine on the activity of the biosynthetic TD of *C. glutamicum* KMV only inhibited by about 50 %, when a high concentration was used. The negative impact of KMV could be compensated by a higher concentration of the substrate L-threonine.

In addition to the KMV experiments, the impact of KIV and KIC on the TD activity was determined. The activities were measured with 40 mM L-threonine as substrate and 20 and 50 mM of KIV or KIC as additional substances contained in the assay mixture. The inhibiting effect of KIV and KIC was weaker than that of KMV in both cases (figure 17). The specific enzyme activity decreased only by about 20 % when 50 mM KIV or KIC were added to the test.

The native biosynthetic TD of *C. glutamicum* has been shown to be inhibited by about 50 % in the presence of 50 mM KMV. For this enzyme several mutated variants have been described, which are feedback-resistant against L-isoleucine (Möckel *et al.*, 1994). To test the resistance of these variants against KMV, plasmids pECM3 *ilvA* V323A, pECM3 *ilvA* D378G and pECM3 *ilvA* H278R L351S harboring three different variants of *ilvA* were transformed into *C. glutamicum* Δ*ilvA*. Cells extracts of these strains were tested on their specific TD activity with 40 mM L-threonine as substrate and with 20 mM or 50 mM KMV as additional substance in the test. Again the results were compared with tests containing 1 mM L-isoleucine. The strains expressing *ilvA* V323A and *ilvA* D378G both reached a specific activity of 2.5 U / mg protein (figure 18), while the third strain had a lower specific activity of about 1.5 U / mg protein. All three variants of the

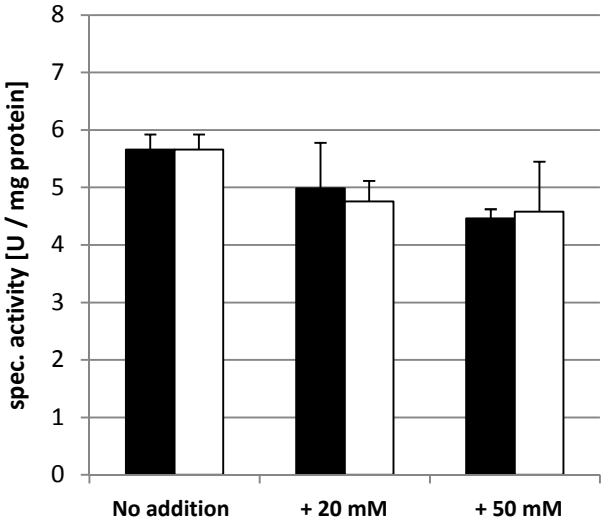

Figure 17: Specific activity of the TD with 40 mM L-threonine as substrate and KIV (black) or KIC (white) as additional substance in the test.

enzyme were reduced in their activity by about 40 % when 50 mM KMV were present. 20 mM KMV only had a small impact on enzyme activity, IlvA H278R L351S was not affected by this concentration at all. The overall performance of the feedback-resistant enzymes in the presence of KMV did not differ significantly to the native enzyme. Astonishingly the feedback-resistant enzymes were also inhibited by 1 mM L-isoleucine. Especially IlvA V323A was reduced by almost 90 % in its activity, which is about the same level as with the native enzyme and therefore it is not suited for strain construction. A KMV production strain has to be transaminase B-negative resulting in L-isoleucine auxotrophy, and therefore L-isoleucine has to be supplemented. A medium concentration of 2 mM L-isoleucine would inhibit IlvA V323A almost completely.

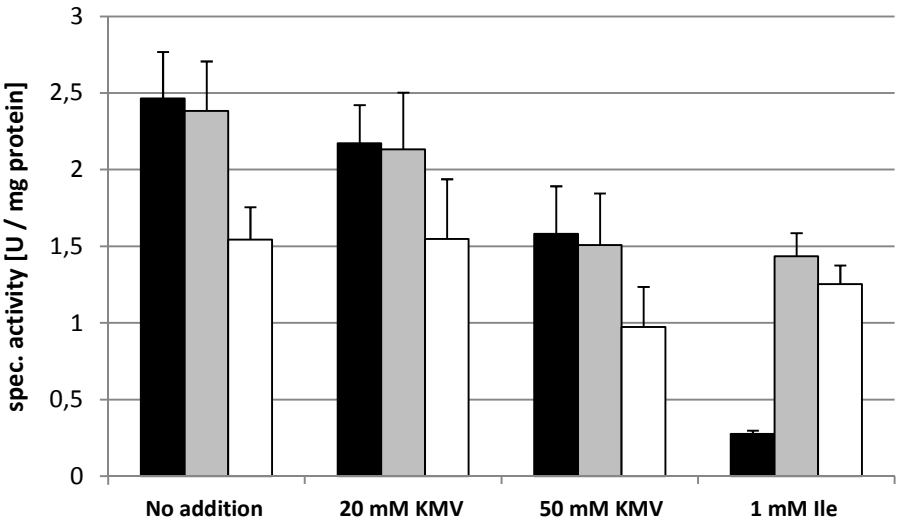

Figure 18: Specific TD activity of *C. glutamicum* Δ*ilvA* (pECM3 *ilvA* V323A) (black), *C. glutamicum* Δ*ilvA* (pECM3 *ilvA* D378G) (grey) and *C. glutamicum* Δ*ilvA* (pECM3 *ilvA* H278R L351S) (white) with 40 mM L-threonine as substrate. Addition of KMV or L-isoleucine as inhibiting substances.

3.4 KMV production with *C. glutamicum*

3.4.1 KMV production with 2-oxobutyrate as a co-substrate

C. glutamicum WT excretes large amounts of L-isoleucine when it is fed with 2-oxobutyrate as co-substrate next to glucose (Eggeling *et al.*, 1987). The addition of 2-oxobutyrate led to a derepression of the *ilvBNCD*-genes so that an efficient L-isoleucine production was possible without overexpression of the respective genes. To evaluate whether it is also possible to synthesize KMV without overexpression of *ilvBNCD* coding for the AHAS, the AHAIR and the DHAD, different strains of *C. glutamicum* were fed with glucose plus 2-oxobutyrate, which is the substrate of the AHAS next to pyruvate. Besides the wild type strain, *C. glutamicum* Δ*ilvE*, *C. glutamicum* Δ*aceE* Δ*ilvE* and *C. glutamicum* Δ*aceE* Δ*ilvE* (pJC4 *ilvBNCD*) were used in these experiments. The *aceE*-deleted strains were employed, because in the L-lysine producing *C. glutamicum* DM1729 the abolishment of the PDHC activity led to a higher excretion of L-lysine (Blombach *et al.*, 2007). This deletion leads to a higher intracellular pool of pyruvate which at least partially is channeled into the biosynthesis branch of the aspartate family of amino acids and thus also towards KMV. Since pyruvate is a substrate of the AHAS it was evaluated whether the deletion of *aceE* is also beneficial for the KMV synthesis.

Both the *C. glutamicum* WT and *C. glutamicum* Δ*ilvE* needed around 30 hours to reach a maximum OD_{600} of about 53 (figure 19 A + B). During this time the 2-oxobutyrate was completely consumed. In parallel the wild type excreted almost 80 mM L-isoleucine, whereas more than 70 mM KMV were observed with *C. glutamicum* Δ*ilvE* after 30 hours of fermentation. This was the first time KMV has been produced using biotechnological methods. Additionally the *ilvE*-deleted strain excreted about 15 mM KIC, which so far has also not been observed as an excreted product in a culture supernatant. The two *aceE*-deleted strains only reached an OD_{600} between 25 and 30 due to the limited amount of acetate in the medium (figure 19 C + D). With these strains also between 70 and 80 mM KMV were observed. However it took a longer time to reach this concentration, the maximal concentration was detected after 48 hours of fermentation. Both strains excreted KIV and pyruvate as byproducts. While *C. glutamicum* Δ*aceE* Δ*ilvE* the excreted more than 30 mM pyruvate, *C. glutamicum* Δ*aceE* Δ*ilvE* (pJC4 *ilvBNCD*) produced up to 40 mM KIV and only 10 mM pyruvate. The overexpression of *ilvBNCD* did not lead to a higher amount of KMV or a faster KMV production and therefore it is probably not necessary.

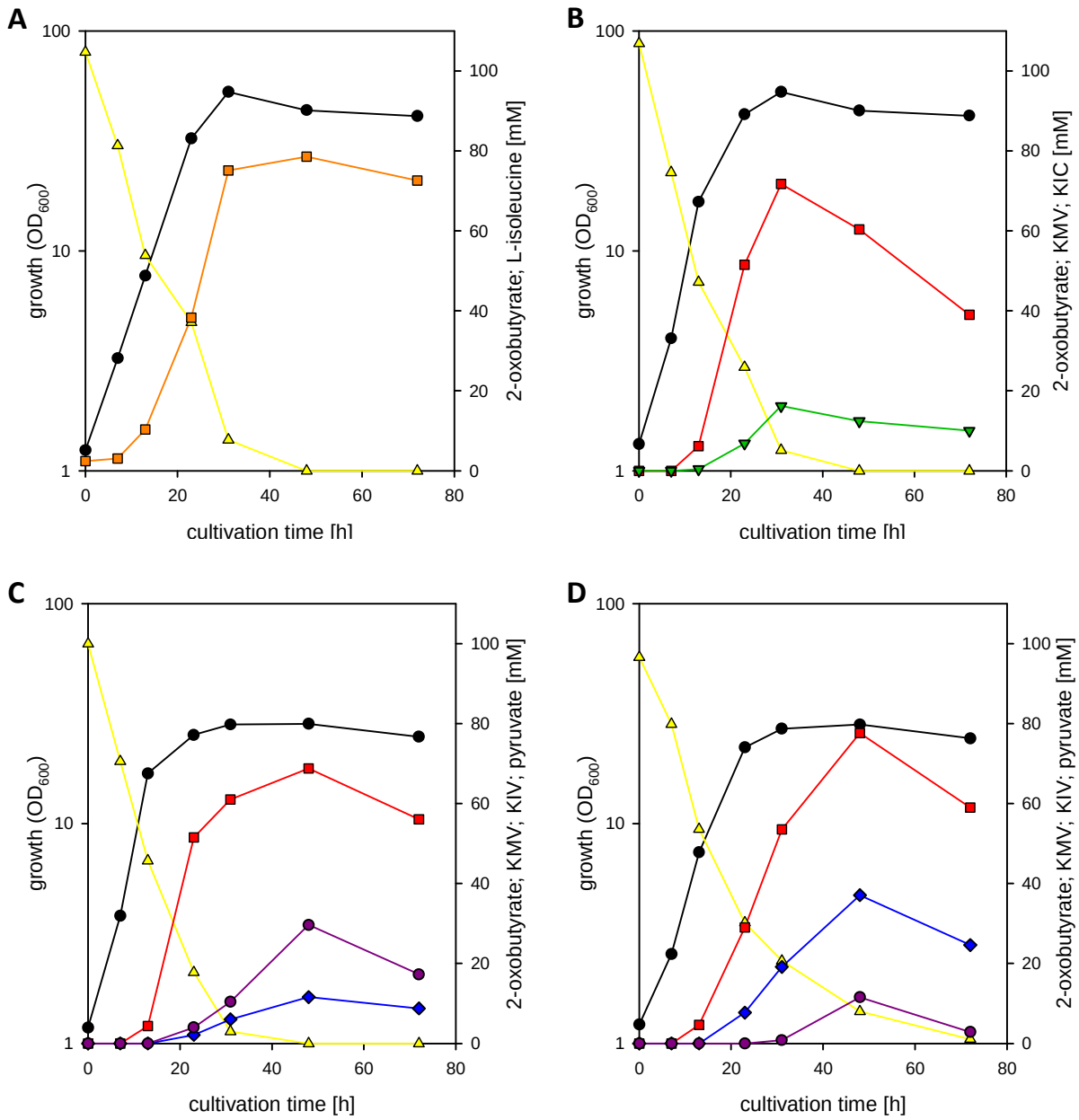

Figure 19: Growth of C. glutamicum WT (**A**), *C. glutamicum* Δ*ilvE* (**B**), *C. glutamicum* Δ*aceE* Δ*ilvE* (**C**) and *C. glutamicum* Δ*aceE* Δ*ilvE* (pJC4 *ilvBNCD*) (**D**) in CgXII-minimal medium + 4 % glucose + L-valine, L-leucine and L-isoleucine (2 mM each) plus addition of 100 mM 2-oxobutyrate. The *aceE*-deleted strains were additionally supplemented with 1 % acetate. The graphs show the growth ● and the concentrations of 2-oxobutyrate △, isoleucine ■, KMV ■, KIC ▼, KIV ◆ and pyruvate ●.

The experiment showed that it is possible to produce KMV on the biotechnological way with *C. glutamicum*. Especially remarkable is the high yield of more than 0.7 mol KMV per mol 2-oxobutyrate. As a result, the focus for the construction of a strain that produces KMV from glucose will be the manipulation of the metabolism in order to increase the intracellular 2-oxobutyrate concentration.

Again it became obvious that KMV is not stable in the culture supernatant. While L-isoleucine remained more or less on the same level in the culture of *C. glutamicum* WT after 2-oxobutyrate was depleted, the KMV-concentration in the other cultures decreased after the maximum concentration was reached. Thus, KMV is not only degraded by growing bacteria, but a production strain also degrades its own product after reaching the stationary phase.

The supernatants of this experiment were also analyzed by gas chromatography to detect if further substances are present that cannot be detected via HPLC. With all four fermentations up to 9 mM 2-methylbutyraldehyde were detected, most of which was already formed during the exponential growth and production phase. 2-methylbutyraldehyde can be formed by the decarboxylation of KMV. The two *aceE*-deleted strains also excreted 3 mM acetoine which is formed in a spontaneous reaction from acetolactate, an intermediate of the L-valine and KIV biosynthesis.

3.4.2 Construction and analysis of a KMV production strain

Since both L-lysine and L-isoleucine share their synthesis pathway down to aspartate semialdehyde, a L-lysine production strain, *C. glutamicum* DM1729 (Georgi *et al.*, 2005), was used as a basis for the construction of a KMV production strain. The strain *C. glutamicum* DM1729 possesses three defined mutations compared to the wild type strain. The anaplerotic enzyme pyruvate carboxylase is deregulated (*pyc*P458S), the aspartate kinase which is strictly inhibited in its activity by L-lysine and L-threonine is present in a feedback-resistant version (*lysC*T311I) and the HOM is present in a less active form (*hom*V59A). The first two mutations are also beneficial for KMV synthesis, whereas the latter one has to be compensated by the overexpression of the hom^{FBR}-gene, coding for a feedback-resistant version of the HOM.

Deletion of *ilvE*

The first step of strain construction was the deletion of the *ilvE*-gene, coding for the transaminase B. In an *ilvE*-deletion strain KMV can no longer be transformed into L-isoleucine and should accumulate. Therefore *C. glutamicum* DM1729 was transformed with the plasmid pK19mobsacB *ilvE*-del that integrates into the genome by homologous recombination and cultivated on kanamycin-containing selective media. In a second step the clones were plated on sucrose-containing agar plates without kanamycin. Sucrose is converted to the toxic metabolite levan by the levan sucrase which is encoded by the *sacB*-gene on the integrated plasmid. Thus, a selective pressure was implied that the plasmid is cut out again by a second homologous recombination. Theoretically there is a 50 % chance either that the original gene is cut out together with pK19mobsacB and thus the deletion was successful or the wild type allele is re-established. To identify clones with the deleted gene a colony PCR was performed with the primer molecules “ilvE1” and “ilvE2” which lead to products of 1820 bps length with the wild type and 1000 bps with the *ilvE*-deleted strain. The *ilvE*-deletion mutant was no longer able to synthesize L-isoleucine and L-leucine. Therefore *C. glutamicum* DM1729 Δ*ilvE* and all of its derivatives had to be supplemented with L-isoleucine, L-leucine and L-valine (2 mM each) when they were cultured in minimal medium. Although L-valine still could be synthesized by the valine-pyruvate transaminase (*avtA*), it was also added to the medium to exclude any effects caused by possibly lower valine availability.

C. glutamicum DM1729 and *C. glutamicum* DM1729 Δ*ilvE* were cultured in CgXII minimal medium with 4 % glucose as carbon source and compared in their growth characteristics and product spectrum. Figure 20 shows representative fermentations for these strains. The growth rate of the *ilvE*-deleted strain was slightly slower than that of the parental strain (0.30 and 0.35

h^{-1}, respectively). Consistent with the lower growth rate, the glucose consumption was also slower by *C. glutamicum* DM1729 Δ*ilvE*. Both strains reached a maximum OD_{600} of around 35. The only metabolites measured in the culture supernatant were L-lysine and very low amounts of L-threonine (< 2 mM). Whereas *C. glutamicum* DM1729 produced between 20 and 25 mM L-lysine the observed concentrations with *C. glutamicum* DM1729 Δ*ilvE* were around 60 % higher with up to 40 mM. Thus, the knock out of the transaminase B somehow lead to a higher flux into the L-lysine biosynthesis branch.

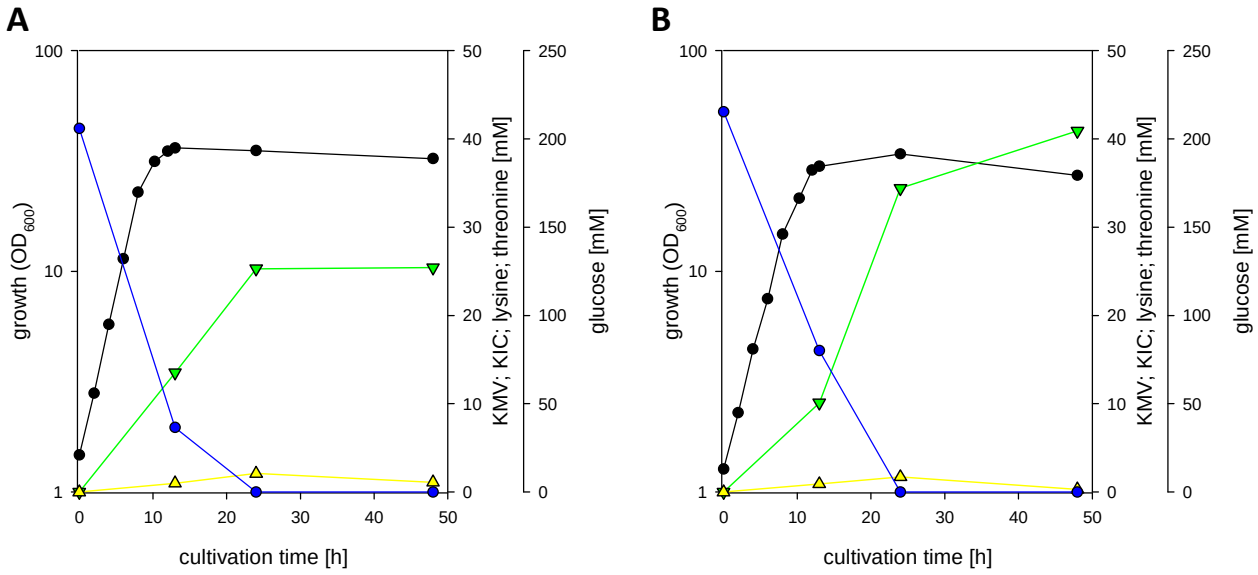

Figure 20: Growth of *C. glutamicum* DM1729 (**A**) and *C. glutamicum* DM1729 Δ*ilvE* (**B**) in CgXII minimal medium with 4 % glucose, 0.5 % BHI and L-valine, L-leucine, L-isoleucine (2 mM each). Documentation of the growth ● , glucose ●, threonine △ and lysine concentration ▽.

Overexpression of *hom*^{FBR}, *thrB*, *thrC* and *ilvA*

The next step in the construction of the KMV-producer was the overexpression of the genes coding for a feedback-resistant version of the HOM (*hom*^{FBR}), the homoserine kinase (*thrB*), the threonine synthase (*thrC*) and the TD (*ilvA*).

First of all *ilvA* was cloned into the vector pMM36ptac. Therefore the coding region of *ilvA* was amplified via PCR using the primer molecules "ilvA_for SmaI" and "ilvA_rev StuI". On this way the ribosomal binding site of the GAP-dehydrogenase gene (*gap*), which is known to work well for the overexpression of genes in *C. glutamicum*, was inserted in front of *ilvA*. The DNA-fragment was digested with the blunt-cutting restriction enzymes *Sma*I and *Stu*I, whose recognition sites were part of the primer molecules. The insert was subsequently ligated into pMM36ptac, which was also cut with *Sma*I. As organism for the amplification of the plasmid *E. coli* XL1 blue was chosen, since this strain expresses the *lac*-repressor LacI^{q} which also represses the ptac promoter of pMM36ptac. This was necessary, because the high level expression of the TD turned out to be lethal for *E. coli* strains not expressing LacI^{q}. In a second step *hom*^{FBR} and *thrB* should be integrated into the vector behind *ilvA*. However this turned out to be not possible, as the only clones that were obtained, contained the genes in the opposite orientation. Since the expression of all genes on a single plasmid was not successful, a second approach was done with two vectors. The plasmids pEK *hom*^{FBR} *thrB thrC* and pMM36ptac *ilvA* were successively transformed into *C. glutamicum* DM1729 Δ*ilvE* via electroporation. The functionality of the plasmids in the resulting strain *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) was ensured by measuring the enzymatic activity of the HOM and the TD. For the HOM a ten-fold higher specific activity was detected (2.49 U/mg of protein compared to 0.24 U/mg of protein in *C. glutamicum* DM1729). With the TD only a five-fold higher activity could be observed with 0.18 and 0.91 U/mg of protein in *C. glutamicum* DM1729 and *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*), respectively. In contrast, a much higher activity was measured if pMM36ptac *ilvA* was the only plasmid transformed into *C. glutamicum* DM1729. In this case a 37-fold higher specific activity (6.80 U/mg of protein) was detected, so the combined overexpression of *ilvA* and *hom*^{FBR}*thrB* proved to be somehow problematic.

C. glutamicum DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) and *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) were cultured in CgXII minimal medium with 4 % glucose as carbon source. The growth of the two strains was very similar (figure 21 A + B). Both strains grew with a growth rate of about 0.23 h^{-1} and reached a maximum OD_{600} of 38. The glucose was consumed after 24 hours. HPLC analysis of culture supernatants revealed for the first time KMV as a product in a culture with glucose as only carbon source, although the concentrations were still low (3 to 5 mM). The measured L-lysine concentration of about 10 mM with both strains

was 75 % lower than with *C. glutamicum* DM1729 Δ*ilvE*, thus, the overexpressed genes led to a redirection of the carbon flux away from L-lysine synthesis. The L-threonine concentration of 6 mM in the culture of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) was slightly higher than in the culture with the strain also expressing *ilvA* (3 mM threonine). This may be a hint on the activity of the TD, which converts L-threonine to 2-oxobutyrate. However, the combined amounts of L-threonine and KMV are much lower than that of the decrease in L-lysine. Thus, obviously there is a bottleneck in the metabolic pathway towards KMV. But it has been shown that KMV synthesis from glucose is possible and if the bottleneck is localized and eliminated also higher concentrations of KMV may be achieved.

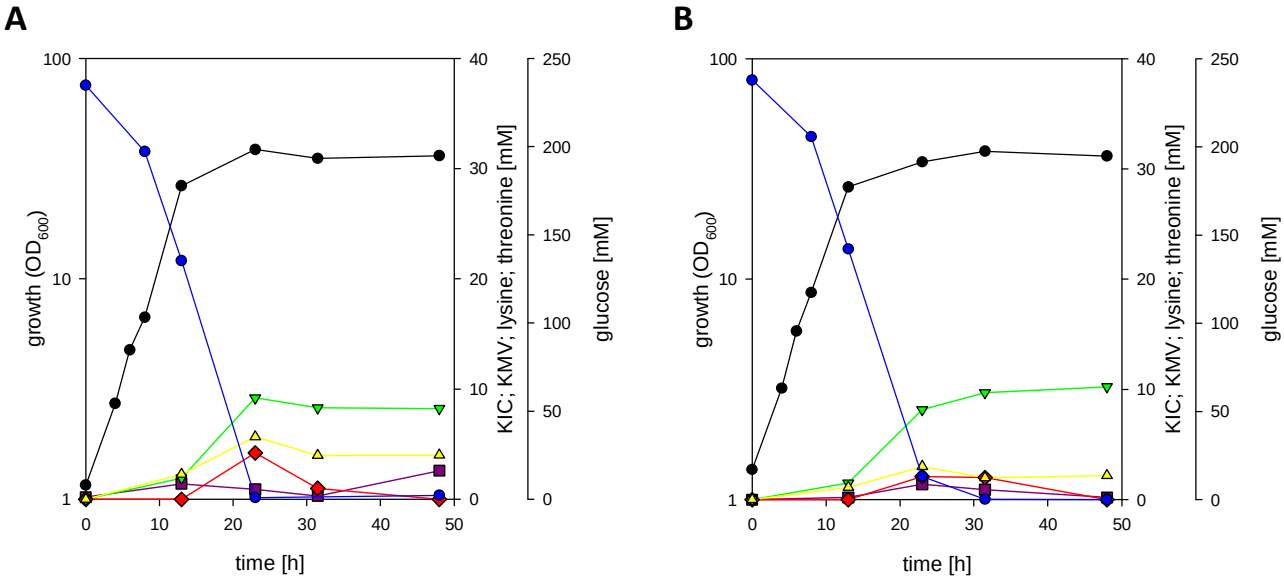

Figure 21: Fermentation of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (**A**) and *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) (**B**) in CgXII minimal medium + 0.5 % BHI + 4 % glucose + 2 mM L-valine, L-leucine, L-isoleucine. Growth ◆, concentration of glucose ●, lysine ▼, threonine △, KMV ◆, and KIC ■.

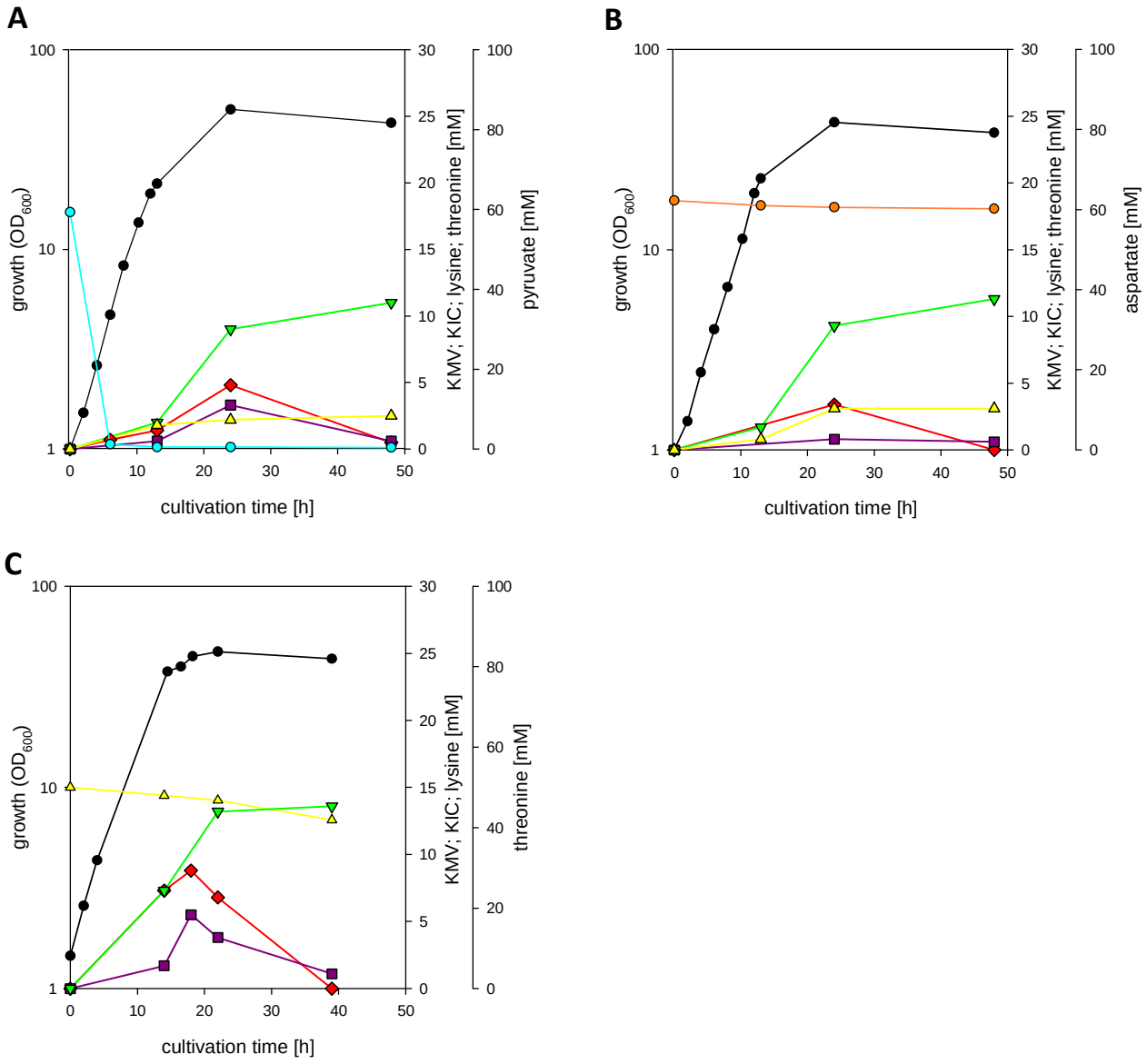

Figure 22: Growth and product analysis of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) in CgXII minimal medium + 0.5 % BHI + 4 % glucose + L-valine, L-leucine, L-isoleucine (2 mM each). Addition of pyruvate (**A**), L-aspartate (**B**) or L-threonine (**C**). Growth ◆, concentration of lysine▽ , KMV◆, KIC■, pyruvate ○, aspartate ●, and threonine△.

The fermentation of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) described before showed that the overexpression of *hom*^{FBR}, *thrB*, *thrC* and *ilvA* led to a redirection of the carbon flux away from L-lysine biosynthesis. However, it also showed that the metabolic pathway is not open for KMV synthesis in a significant dimension. To find the location of this limitation further growth experiments were performed with the addition of several KMV precursor metabolites to the medium. Pyruvate, which is next to oxaloacetate the main precursor of KMV synthesis, was completely consumed by the bacteria in 6 hours (figure 22 A). However, there was no change in the product spectrum in the presence of pyruvate. At least part of the pyruvate was used for biomass formation, since the culture reached a higher OD_{600} of 50 when compared to the other cultures (figure 22 B + C) supplemented with L-aspartate and L-threonine (OD_{600} of 43). L-aspartate was not absorbed by the bacteria (figure 22 B), while the concentration of L-threonine dropped by 10 mM during 40 hours (figure 22 C). L-aspartate also had no effect on excreted metabolites. Only with L-threonine differences were observed, the KMV concentration increased to 9 mM. However it was not possible gain new conclusions on the bottleneck in the KMV synthesis pathway, because L-threonine was metabolized by the bacteria in relatively small amounts.

3.4.3 KMV production under limitation of branched-chain amino acids

In a further fermentation of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) the amino acids L-valine, L-leucine and L-isoleucine were replaced by the complex substance BHI as medium ingredient. The culture grew with a growth rate of 0.27 h^{-1} for eight hours (figure 23 A), which is slightly faster than the culture which was supplemented with amino acids (figure 20 B). Afterwards growth slowed down until a maximum OD_{600} of 50 was reached when glucose in the medium was depleted. The most remarkable result of this fermentation was the increase of the KMV concentration to 15 mM after 24 hours. Additionally 18 mM L-lysine and 6 mM KIC were measured in the culture supernatant. This led to the assumption that the supplemented amino acids L-valine, L-leucine and L-isoleucine had an inhibiting effect on KMV synthesis. As a consequence, *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) was cultured in CgXII minimal medium with 4 % glucose without any further additions to complement the *ilvE* deletion. Surprisingly, and in contrast to *C. glutamicum* DM1729 Δ*ilvE*, *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) was able to grow in this medium (figure 23 B). Under these conditions more than 35 mM KMV were detected in the culture supernatant after 48 hours. The L-lysine excretion decreased to about 8 mM and L-threonine was no longer present in detectable amounts. This strengthens

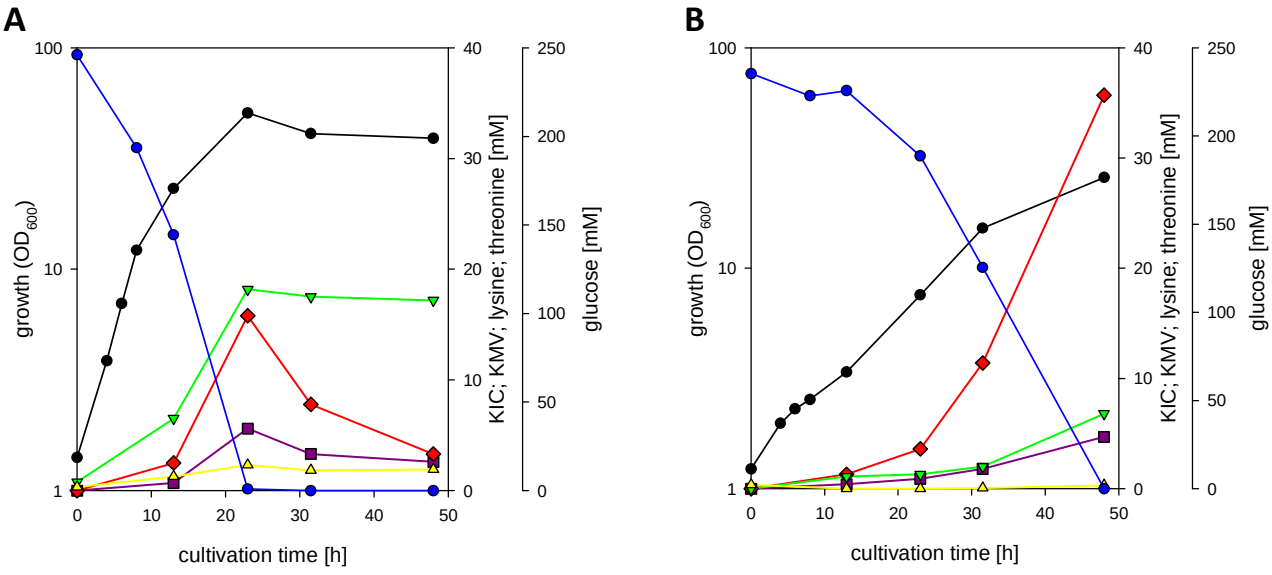

Figure 23: Fermentation of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) in CgXII minimal medium + 0.5 % BHI + 4 % glucose (**A**) and CgXII minimal medium + 4 % glucose (**B**). Growth ●, concentration of glucose ●, lysine ▼, threonine ▲, KMV ◆ and KIC ■.

the assumptions that the supplementary amino acids have a negative effect on KMV synthesis. Also the concentrations of byproducts could were reduced in the fermentation without additions.

To examine the inhibiting effect of the supplemented amino acids in more detail, further growth experiments were performed with *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) with the addition of L-valine, L-leucine and L-isoleucine alone and in all possible combinations.

A culture without supplementary amino acids was used as a reference (figure 24 A). This culture grew with a growth rate of 0.07 h^{-1} up to a maximal OD_{600} of 28 in 48 hours. The highest product concentrations in the culture supernatant were detected 56 hours after inoculation with 23 mM KMV and 14 mM L-lysine. The addition of L-valine had a strong inhibiting effect both on growth and production of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*). The culture doubled about twice in the first twelve hours after inoculation (figure 24 B). Then the growth stagnated until a second exponential growth phase started after 48 hours, when L-valine was depleted. After this point especially the high level of L-lysine excretion is remarkable. The culture supplemented with L-leucine had a slightly lower growth rate compared to the not-supplemented culture with 0.05 and 0.07, respectively (figure 24 C). Additionally, growth slowed down after 30 hours and therefore the culture did not reach an OD_{600} of 20 before 72 hours of growth, whereas the culture without additions reached an OD_{600} of 25 after 48 hours. However, the L-leucine-supplemented culture showed the highest KMV excretion (30 mM), a very low L-lysine production of 7 mM and nearly no L-threonine formation. Also no KIC was detected in the culture supernatant, whereas at least 3 mM KIC were measured with all other cultures. In contrast, the addition of L-isoleucine led to a recovery of the growth rate on a relatively high value of 0.20 h^{-1} (figure 24 D). However, L-isoleucine almost completely anticipates the synthesis of KMV. Only 5 mM of KMV were observed, while the concentrations of L-lysine and L-threonine with 18 and 3 mM were higher compared to the not-supplemented culture, where 14 and 1 mM were detected.

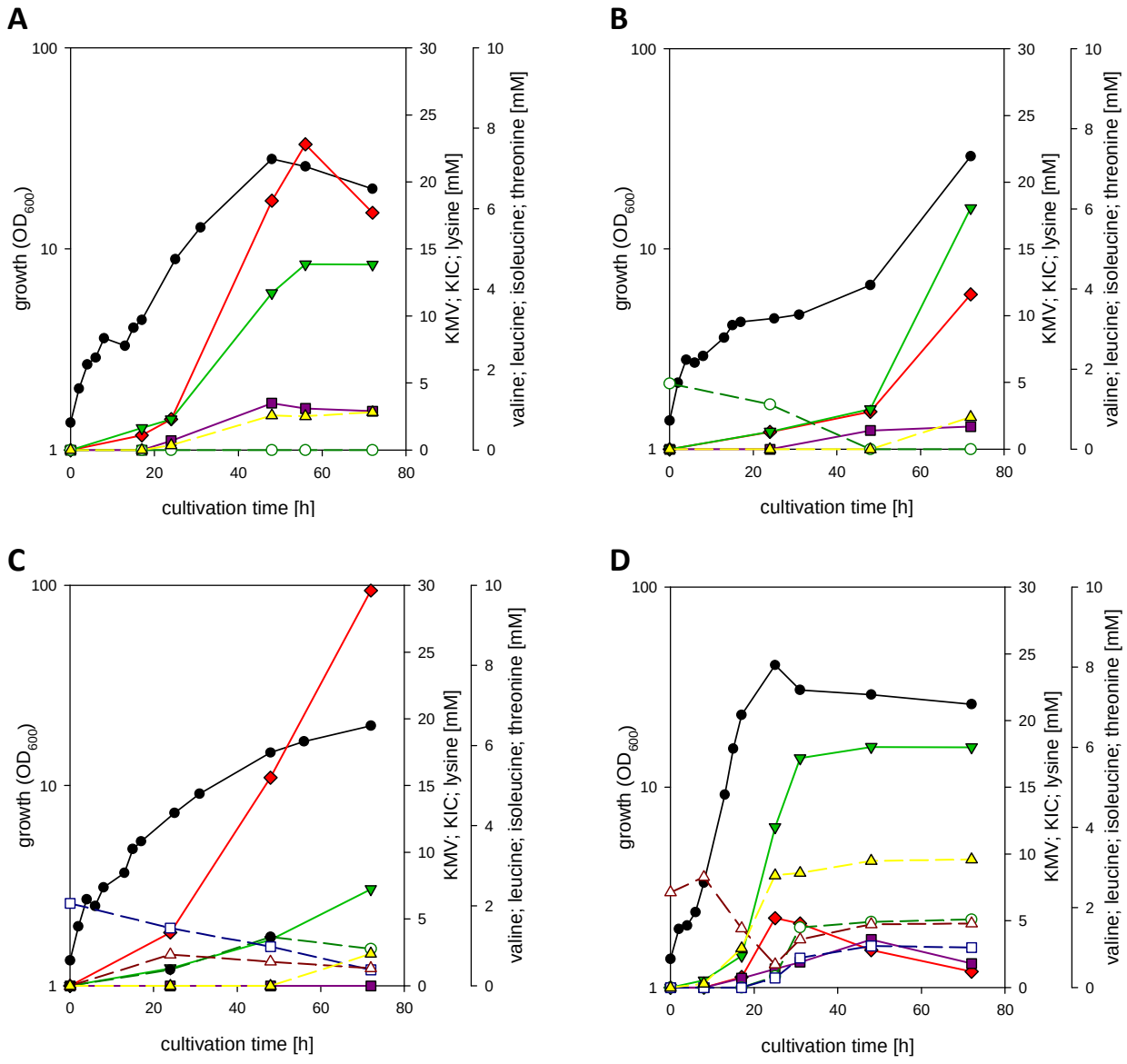

Figure 24: Growth of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) in CgXII minimal medium + 4 % glucose without additions (**A**), with 2 mM L-valine (**B**), with 2 mM L-leucine (**C**), with 2 mM L-isoleucine (**D**). Continues lines refer to the primary, broken lines to the secondary Y-axis. Growth ◆ , concentration of lysine ▼, threonine △ , KMV ◆ , KIC ■, valine ○, leucine □, isoleucine △.

Further experiments were performed with different combinations of two amino acids. A culture with L-valine and L-leucine grew up to an OD_{600} of 4 in the first 15 hours after inoculation (figure 25 A). Afterwards the culture stagnated on this level and growth did not recover during 72 hours. Also the amount of the supplemented amino acids did not decrease during this period of time and no significant production took place. Cultures with L-valine and L-isoleucine had a growth rate of 0.14 h^{-1} during the exponential growth phase (figure 25 B) which is lower than in the culture supplemented with L-isoleucine alone but faster than the culture without additions. Thus, the growth-restoring effect of L-isoleucine seems to be dominant, but L-valine weakens this effect. Similar to the L-isoleucine-supplemented culture, only small amounts of KMV (5 mM) but high concentrations of L-lysine, L-threonine and KIC were observed (15, 3.5 and 3.5 mM, respectively). Cultures with L-leucine and L-isoleucine grew with a growth rate of 0.20 h^{-1} up to an OD_{600} of 38 which is identical to cultures supplemented with L-isoleucine alone (figure 25 C). Also the measured concentrations of KMV, L-lysine and L-threonine were in the same range. The highest growth rate of 0.23 h^{-1} was observed, when *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) was grown in the presence of all three branched-chain amino acids (figure 25 D). Similar to the other cultures containing L-isoleucine, 5 mM KMV, 15 mM L-lysine and 4 mM L-threonine were measured. One effect which was observed with most cultures was that the supplemented amino acids were consumed by the bacteria and to some extent excreted again, when the stationary phase was reached. Additionally to the metabolites represented in figures 23 and 24 between 2 and 4 mM L-glutamate were detected in all cultures and up to 6 mM L-alanine were measured in supernatants of cultures supplemented with L-isoleucine and one or both of the other branched-chain amino acids.

By alteration of the medium composition the KMV production of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) has been increased from 5 mM in a culture supplemented with all three branched-chain amino acids to 30 mM in cultures only supplemented with L-leucine. Furthermore with L-isoleucine an inhibiting agent of the KMV synthesis has been identified. The impact of L-isoleucine seems to be dominant over the other two amino acids and strongly anticipates KMV-synthesis. Only in the absence of this amino acid KMV-concentrations higher than 5 mM could be achieved. L-valine on the other hand had a strong inhibiting effect on the growth of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*)(pMM36ptac *ilvA*). Exponential growth only could be observed after all L-valine in the medium was depleted.

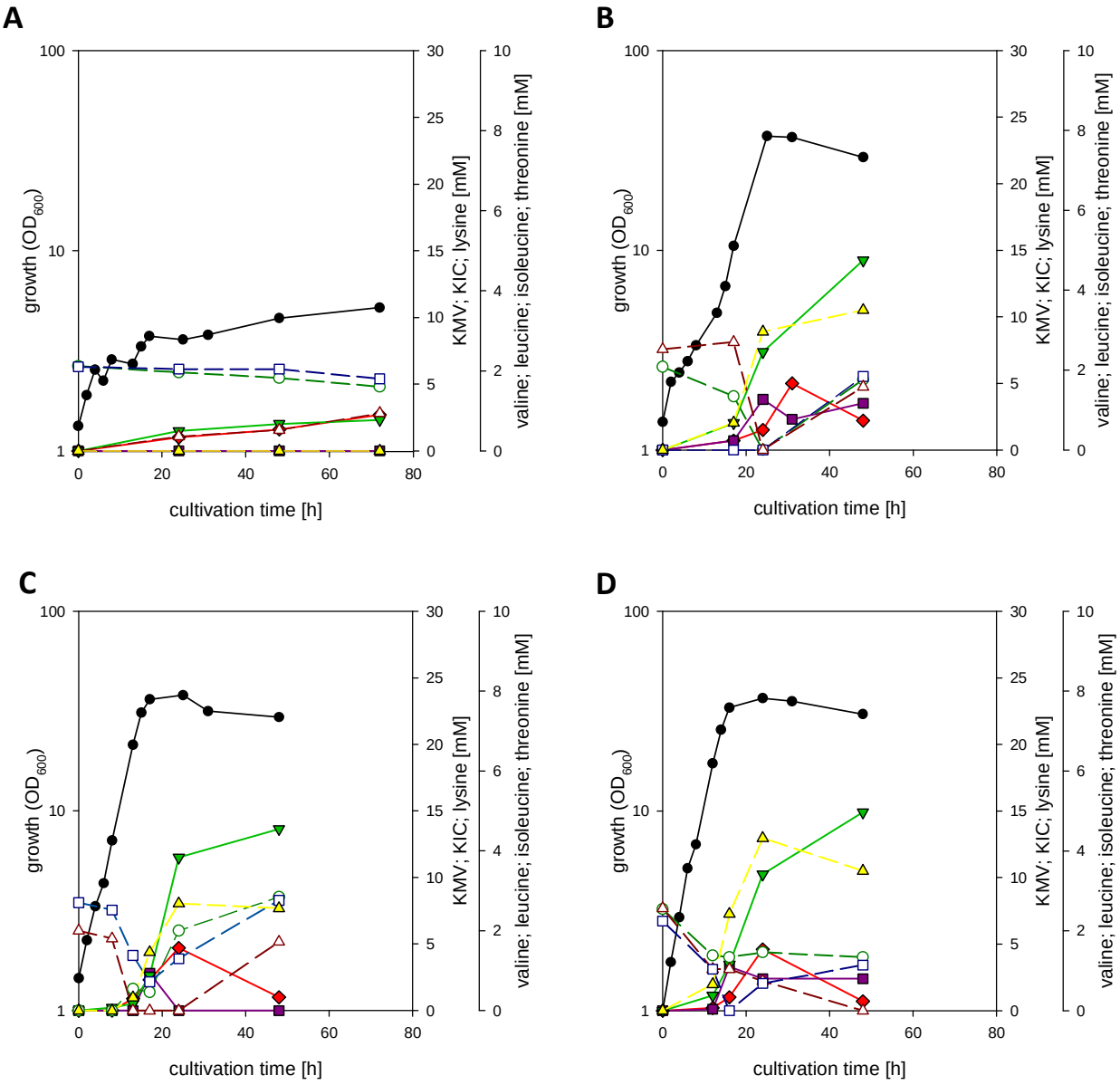

Figure 25: Growth of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) in CgXII minimal medium + 4 % glucose with L-valine and L-leucine (2 mM each) (**A**), L-valine and L-isoleucine (2 mM each) (**B**), L-leucine and L-isoleucine (2mM each) (**C**), L-valine, L-leucine and L-isoleucine (2 mM each) (**D**). Continues lines refer to the primary, broken lines to the secondary Y-axis. Growth ◆, concentration of lysine ▽, threonine △ , KMV ◆, KIC ■, valine ○, leucine □, isoleucine △.

Since the expression of the AHAS is known to be repressed by L-valine via attenuation (Morbach *et al.*, 2000) the activity of the enzyme was measured before and after L-valine-depletion, to evaluate, if a inhibition of the AHAS could be responsible for the growth inhibition. In a culture harvested 24 hours after incubation an AHAS-activity of 333 ± 15 mU / mg of protein was observed. The activity increased to 836 ± 47 mU / mg of protein in the later exponential growth phase (72 hours after inoculation). So there was a 2.5-fold increase of AHAS-activity after valine-depletion, but the activity during the lag-phase was still about 2.5-fold higher than in *C. glutamicum* WT, where 133 ± 8 mU / mg of protein were detected. Thus, a repression of *ilvBN* expression by L-valine cannot be the reason for the growth inhibition in the presence of this amino acid.

3.4.4 Fed-batch fermentation with glucose and 2-oxobutyrate

To ensure that the limited KMV production of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) is not caused by an inhibition of the AHAS, the AHAIR or the DHAD, the strain was also fed with 2-oxobutyrate in addition to glucose. The strain was grown in CgXII-minimal medium + 0.1 % yeast extract + 4 % glucose with the addition of 100 mM 2-oxobutyrate. To evaluate whether a further synthesis of KMV is limited at a higher concentration another 50 mM 2-oxobutyrate were added after 24 hours and after 48 hours of fermentation. Additional 2 % glucose was added after 48 hours, since the initial 4 % glucose was almost depleted at this point of time. Under these conditions *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) reached a maximal OD_{600} of 32 after 56 hours (figure 26). The 2-oxobutyrate was consumed very fast in the first ten hours of the experiment. Later the consumption slowed down, after 24 hours some 2-oxobutyrate remained in the supernatant of the culture. At this time only 18 mM KMV had been excreted into the medium, which means that most of the 2-oxobutyrate has been stored intracellularly or it has been degraded. The second feed of 50 mM 2-oxobutyrate was absorbed completely during the next 24 hours. In parallel the KMV concentration in the supernatant increased to about 110 mM. Another feed of 50 mM 2-oxobutyrate after 48 hours was also consumed until the end of the experiment, which led to a final KMV concentration of 150 mM after 56 hours. Although the bacteria still consumed glucose and 2-oxobutyrate until 72 hours, no further growth and KMV-formation appeared. In addition to the KMV, the bacteria excreted 18 mM L-lysine and 8 mM L-threonine. In the early stage of the formation up to 6 mM KIC could be measured, but the concentration decreased again till the end of the experiment. The experiment showed that the production of higher concentrations of KMV

is possible. However, *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) ceased to grow and produce KMV after 56 hours, only the concentrations of the byproducts increased. Thus, 150 mM KMV may be a limiting concentration that prevents further KMV synthesis.

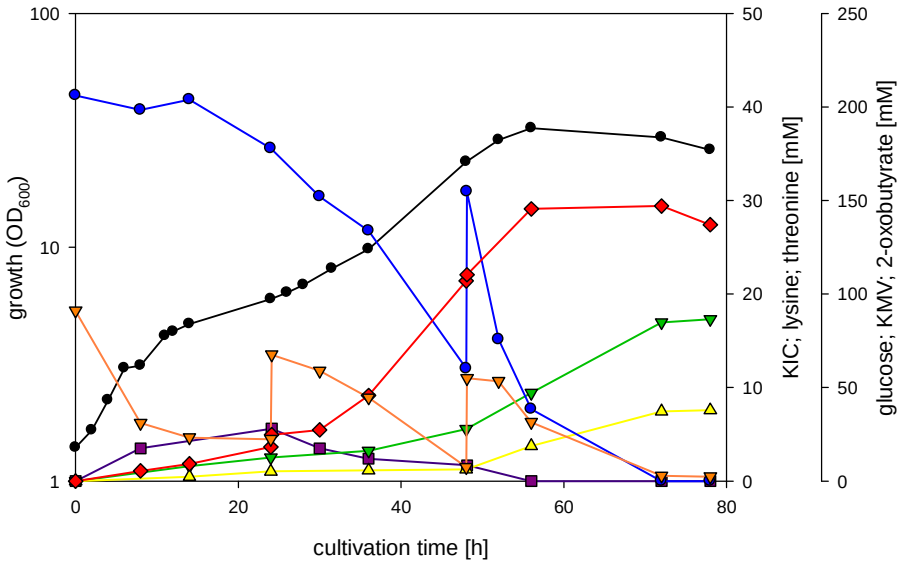

Figure 26: Fed-batch fermentation of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) in CgXII-minimal medium + 0.1 % yeast extract + 4 % glucose with repeated feeding of 2-oxobutyrate. Growth ◆, concentration of lysine ▽, threonine △ , KMV ◆, KIC ■, 2-oxobutyrate ▽ and glucose ●.

3.4.5 Fermentation of the KMV production strain with homoserine as second substrate

To analyze the flux through the KMV biosynthesis pathway beginning with homoserine *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) was cultured in minimal medium with glucose and homoserine as carbon sources. Since the only branching point towards L-methionine is strictly regulated (Rey *et al.*, 2003) the homoserine should largely be transformed to KMV. When homoserine was added to the medium *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) was no longer able to grow in minimal medium. Therefore 0.1 % yeast extract was added and comparative fermentations were made with and without addition of 50 mM homoserine (figure 27 A and B). No differences were observed in the growth behavior and glucose consumption of the different cultures. The added homoserine was absorbed almost completely by the bacteria during 48 hours, less than 10 mM remained in the supernatant. The highest KMV concentration (23 mM) was detected in the culture with homoserine, an increase of 8 mM compared to the culture without homoserine. The L-lysine concentration also increased due to the addition of homoserine compared to the culture without homoserine (21 and 13 mM, respectively), same as the L-threonine concentration (1 and 4 mM).

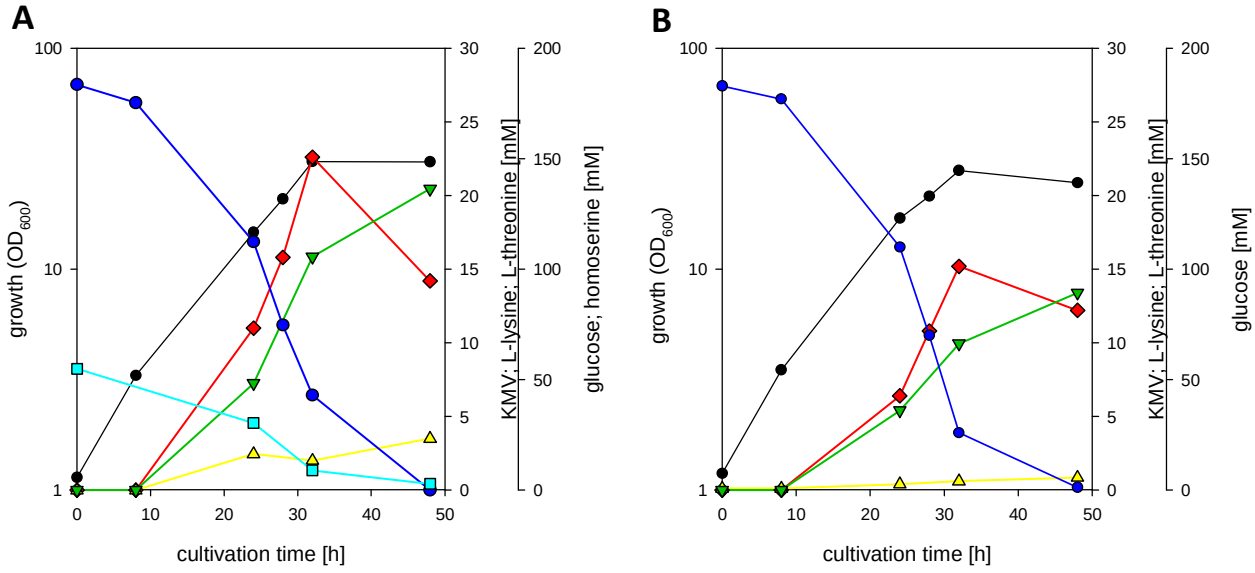

Figure 27: Fermentation of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) in CgXII minimal medium + 0.1 % yeast extract + 4 % glucose. With (**A**) and without (**B**) addition of 50 mM homoserine. Growth ◆ , concentration of lysine ▽, threonine △,KMV ◆, homoserine □and glucose ●.

Thus, the homoserine was only partially converted to KMV, since the amount of absorbed homoserine is much larger than the produced KMV. The increase of L-lysine and L-threonine excretion hint on a inhibition of some of the L-threonine synthesis enzymes.

3.4.6 Use of a feedback-resistant variant of the TD in the KMV production strain

The results obtained with the KMV production strain *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) supplemented with different combinations of the branched-chain amino acids indicated, that L-isoleucine is responsible for the inhibition of KMV synthesis. An enzyme known to be strictly regulated by L-isoleucine is the TD (IlvA). Also the high L-threonine excretion of L-isoleucine-supplemented cultures was a hint on a limitation of the TD reaction. Therefore a feedback-resistant version of the TD was introduced in the production strain by transformation of the plasmid pECM3 *ilvA* D378G (Möckel *et al.*, 1994).

The newly constructed strain *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pECM3 *ilvA* D378G) was cultured in CgXII medium with 0.5 % BHI, 4 % glucose and L-valine, L-leucine and L-isoleucine (2 mM each) in comparison to the KMV-production strain with plasmid pMM36ptac *ilvA* instead of pECM3 *ilvA* D378G. The strain expressing the feedback-resistant TD grew with a lower growth rate of 0.24 h^{-1} (figure 28 A) compared to 0.30 h^{-1} with the old strain (figure 28 B) and consistently, the glucose consumption was slower. Both strains reached a similar maximum OD_{600} of around 50. The amount of produced KMV was only slightly higher with 5 mM compared to 3 mM, while the L-lysine excretion was lower. Only the L-threonine concentration was significantly lower with 1 mM compared to 4.5 mM in the strain expressing native *ilvA*. Both strains were also fermented in medium without the branched-chain amino acids but with 0.5 % BHI (figure 28 C + D), since the strain expressing *ilvA* D378G was not able to grow in minimal medium without any supplementation. Again the strain expressing *ilvA* D378G grew slower with 0.16 h^{-1} compared to 0.20 h^{-1}. With both strains the growth rate decreased after an OD_{600} of about 20 was reached and the maximal OD_{600} was about 45. In the supernatants of both strains about 20 mM KMV were measured and the L-lysine and L-threonine concentration again was slightly lower with *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pECM3 *ilvA* D378G). In summary the use of the feedback resistant version of the TD did not lead to a significantly higher KMV synthesis both in the presence or absence of L-isoleucine, only the lower L-threonine excretion indicated a higher TD activity.

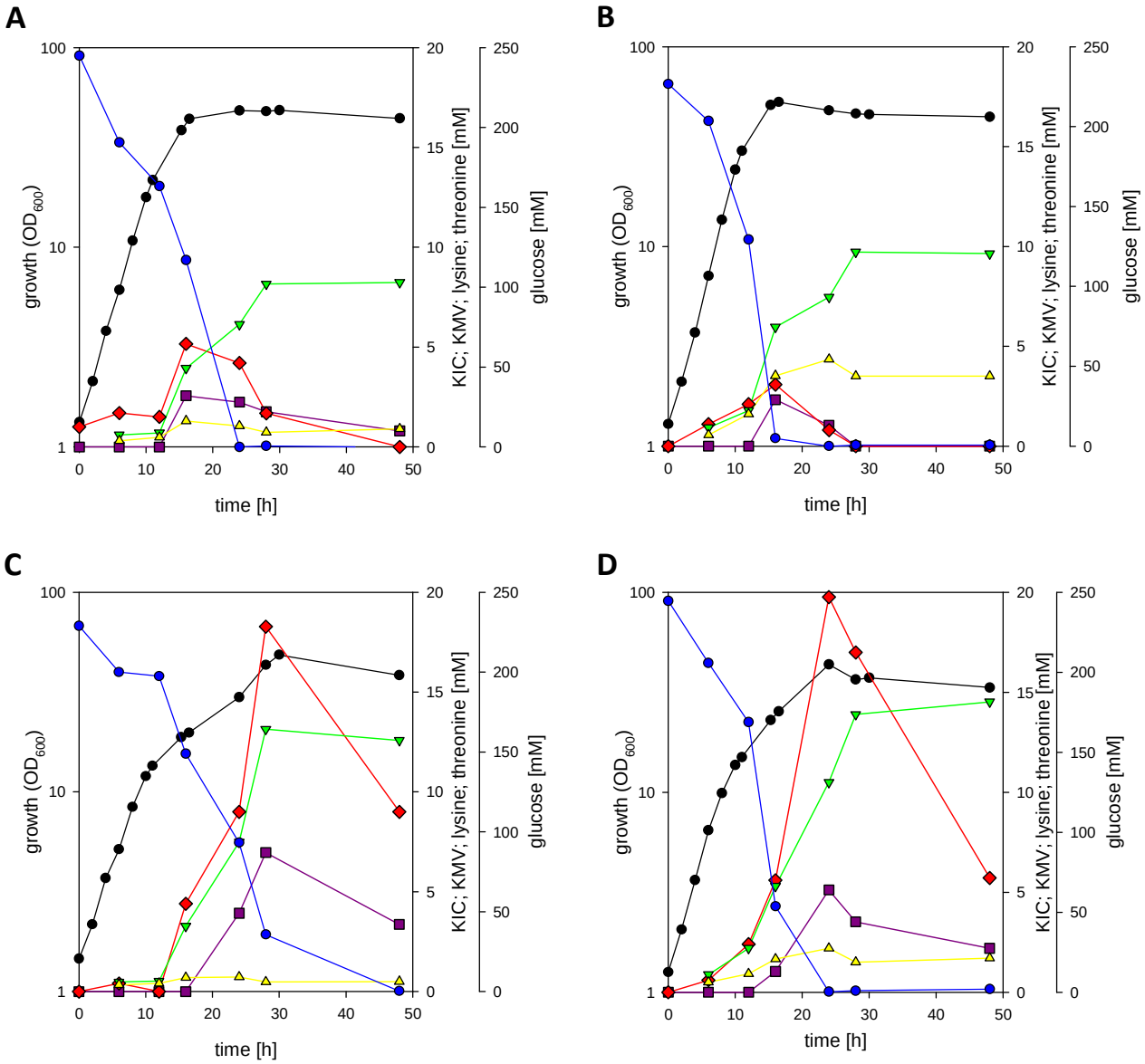

Figure 28: Growth and product formation of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pECM3 *ilvA* D378G) (**A + C**) and *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) (**B + D**) in CgXII medium + 0.5 % BHI + 4 % glucose + L-valine, L-leucine, L-isoleucine (2 mM each) (**A + B**) or in CgXII medium + 0.5 % BHI + 4 % glucose (**C + D**). Growth ◆, concentration of L-lysine ▽, L-threonine △, KMV ◆, KIC ■ and glucose ●.

The activity of the feedback-resistant TD IlvA D378G was measured with increasing concentrations of L-isoleucine to ensure that the enzyme is functional as expected. As shown in figure 29, the overall TD activity in *C. glutamicum* Δ*ilvA* (pMM36 *ilvA*) was more than twice as high as in *C. glutamicum* Δ*ilvA* (pECM3 *ilvA* D378G), when no L-isoleucine was added to the test. The native enzyme is severely inhibited by L-isoleucine. Even at a low L-isoleucine concentration of 0.5 mM the activity was reduced by more than 50 % and with 5 mM L-isoleucine only 5 % of the specific activity was detected. The feedback-resistant enzyme showed a higher resistance against up to 1 mM L-isoleucine, but at higher concentrations the activity went down to the same low level as with the native enzyme. With 2 mM L-isoleucine as it was used as medium ingredient, the two enzymes do not have a significant difference in their specific activity. Thus, the expression of *ilvA* D378G in the KMV production strain does not offer a great advantage to the use of the native TD.

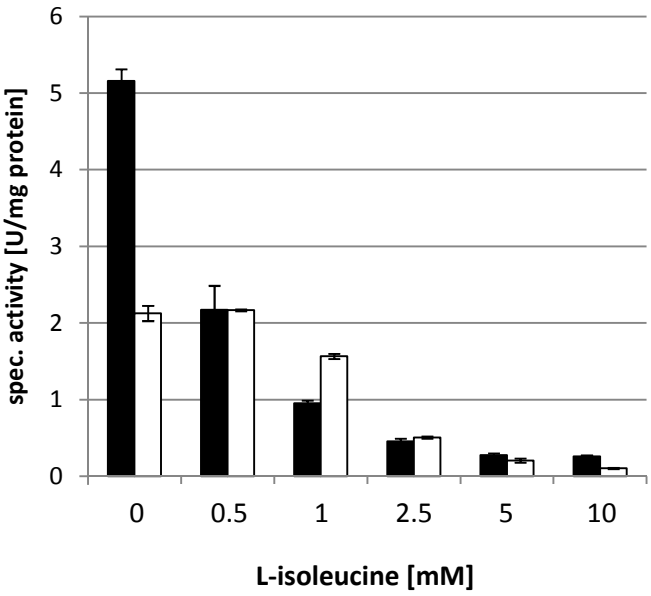

Figure 29: Specific TD activity of *C. glutamicum* Δ*ilvA* (pMM36 *ilvA*) (black bars) and *C. glutamicum* Δ*ilvA* (pECM3 *ilvA* D378G) (white bars) in the presence of increasing concentrations of L-isoleucine.

3.4.7 Use of the catabolic TD (*tdcB*) of *E. coli*

The feedback-resistant version of the TD IlvA D378G did not possess a sufficient resistance against L-isoleucine as shown in the previous chapter. The catabolic TD of *E. coli* (encoded by *tdcB*) was considered as an alternative, because this enzyme is described to have a very high resistance against L-isoleucine (Guilouet *et al.*, 1999). The coding sequence of *tdcB* was amplified from chromosomal DNA of *E. coli* K12 via PCR using the primers "tdcB (*E. coli*) hin RBS" and "tdcB (*E. coli*) rück PstI" and ligated into the expression vector pMM36ptac via the restriction sites of *Sma*I and *Pst*I. The generated plasmid (pMM36ptac *tdcB*) was transformed into *C. glutamicum* Δ*ilvA* via electroporation and the specific enzyme activity of TdcB was measured. The same enzyme assay as for the measurement of the activity of the biosynthetic TD of *C. glutamicum* was used with the addition of 1 mM AMP. TdcB needs AMP as a cofactor to form a stable homotetramere (Whanger *et al.*, 1968). Without AMP only a relatively low activity of 0.39 mU / mg of protein could be detected which is less than one quarter of the activity measured with 1 mM AMP (1.69 ± 0.05 U / mg of protein). As shown in figure 30, the activity of the catabolic TD was not affected by L-isoleucine in concentrations of up to 50 mM. Also with a high KMV concentration of 50 mM the specific enzyme activity decreased less than 10 %. Thus, the use of the catabolic TD of *E. coli* is a promising alternative for IlvA in the KMV production strain.

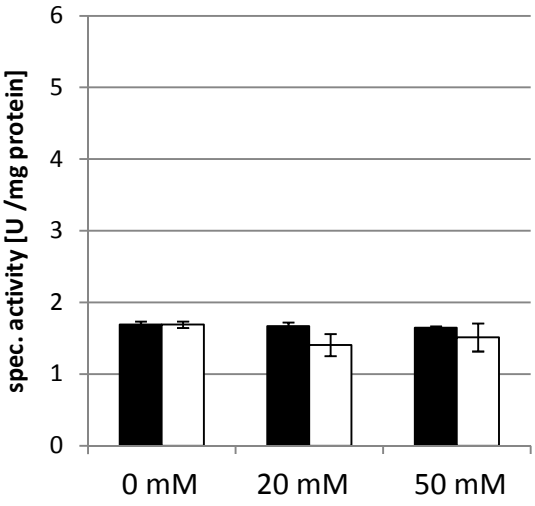

Figure 30: Specific TD activity of *C. glutamicum* Δ*ilvA* (pMM36ptac *tdcB*) in the presence of different concentrations of L-isoleucine (black bars) or KMV (white bars).

Expression of *tdcB* in the KMV-production strain

The catabolic TD of *E. coli* was introduced into the KMV production strain by the transformation of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) with the plasmid pMM36ptac *tdcB*. The resulting strain *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *tdcB*) was cultured in CgXII minimal medium with 4 % glucose as carbon source with and without addition of 0.1 % yeast extract. The culture grew with a growth rate of 0.08 h^{-1} and reached a final OD_{600} of 35 after 44 hours (figure 31). The highest concentration of KMV and L-lysine were detected after 44 hours (25 mM each). Additionally 5.5 mM KIC and 3.5 mM L-threonine were detected in the culture supernatants. Thus, in comparison to the KMV production strain expressing *ilvA*, *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *tdcB*) produced less KMV but almost the double concentration of L-lysine.

Since the excreted KMV-concentration in the culture supernatant of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *tdcB*) was not higher than with the strain expressing *ilvA*, the functionality of the catabolic TD was tested in the production strain by measuring the enzyme activity. A specific TD activity of 214 mU / mg protein was detected which is 2.5 fold higher than the activity measured with *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (80

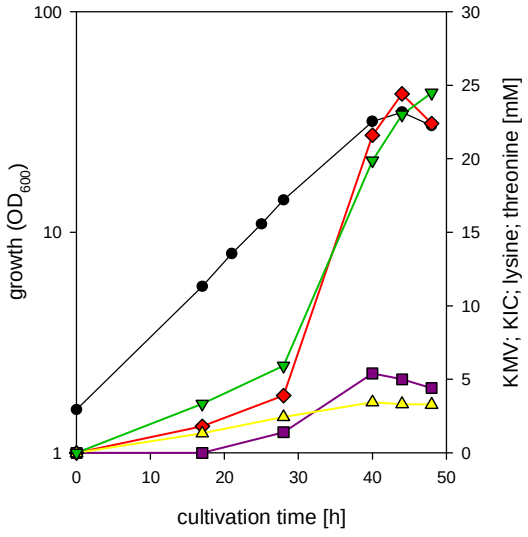

Figure 31: Growth and product analysis of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *tdcB*) in CgXII minimal medium + 4 % glucose. Growth ●, concentration of L-lysine ▼, L-threonine △, KMV ◆ and KIC ■.

mU / mg protein). However the activity was much lower than with *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*), expressing the gene for the native TD of *C. glutamicum* (0.91 mU / mg protein). Thus, the overexpression of *tdcB* does somehow not work as well as the expression of *ilvA* in the KMV production strain.

3.4.8 Flux reduction into L-lysine biosynthesis

All constructed KMV production strains have in common that they still excrete L-lysine as a major byproduct. Since the L-lysine biosynthesis competes with the KMV synthesis branch for the precursor molecule aspartate semialdehyde, it is advisable to reduce the flux into L-lysine. The L-lysine biosynthesis pathway cannot be completely abolished, because it is not possible to supplement the precursor of L-lysine, diaminopimelate, which is necessary for cell wall synthesis. Therefore the activity of the dihydrodipicolinat synthase (encoded by *dapA*), the initial enzyme of the L-lysine branch, was reduced. For this purpose in a co-work with Dennis Hell plasmids based on the integration vector pK19mobsacB were constructed that allow the exchange of the promoter of *dapA* against weaker promoters (Hell, 2010), using the promoter sequences described by Vasicova *et al.* (1999). The three plasmids pK19mobsacBdapA-Prom:C5, pK19mobsacBdapA-Prom:C13 and pK19mobsacBdapA-Prom:B31 were each transformed into *C. glutamicum* DM1729 Δ*ilvE* via electroporation and plated on selective agar plates. By plating them on sucrose-containing plates the plasmids were forced to be cut out via homologous recombination leaving the *dapA*-gene with the exchanged promoter in the genome, same as described in the chapter 3.3.2. A successful promoter exchange was determined by a colony PCR using the primers “dapA-Prom Nachweis” and “dapA rev BamHI” which leads to a 578 bp long fragment with positive clones.

The constructed strains *C. glutamicum* DM1729 Δ*ilvE dapA*-Prom:C5, *C. glutamicum* DM1729 Δ*ilvE dapA*-Prom:C13 and *C. glutamicum* DM1729 Δ*ilvE dapA*-Prom:B31 were culture in CgXII minimal medium with 4 % glucose in comparison to *C. glutamicum* DM1729 Δ*ilvE* in order to find out, whether the promoter exchange led to a reduced L-lysine production. As shown in figure 32 A, the promoter exchange did not affect the growth. All strains grew with a growth rate of 0.24 h^{-1} up to a final OD_{600} of about 40. Figure 32 B shows the L-lysine concentrations that were measured in the culture supernatants after 24 hours. *C. glutamicum* DM1729 Δ*ilvE* reached a concentration of 45 mM L-lysine while the concentrations of all mutant strains were lower. The use of the B31-promoter reduced the L-lysine concentration slightly by about 15 %, with the C13-promoter the concentration was 30 % lower and with the weakest promoter (C5) a more

than 60 % lower L-lysine concentration was measured. The reduction of the L-lysine excretion was consistent with the described promoter strengths (Vasicova *et al.*,1999).

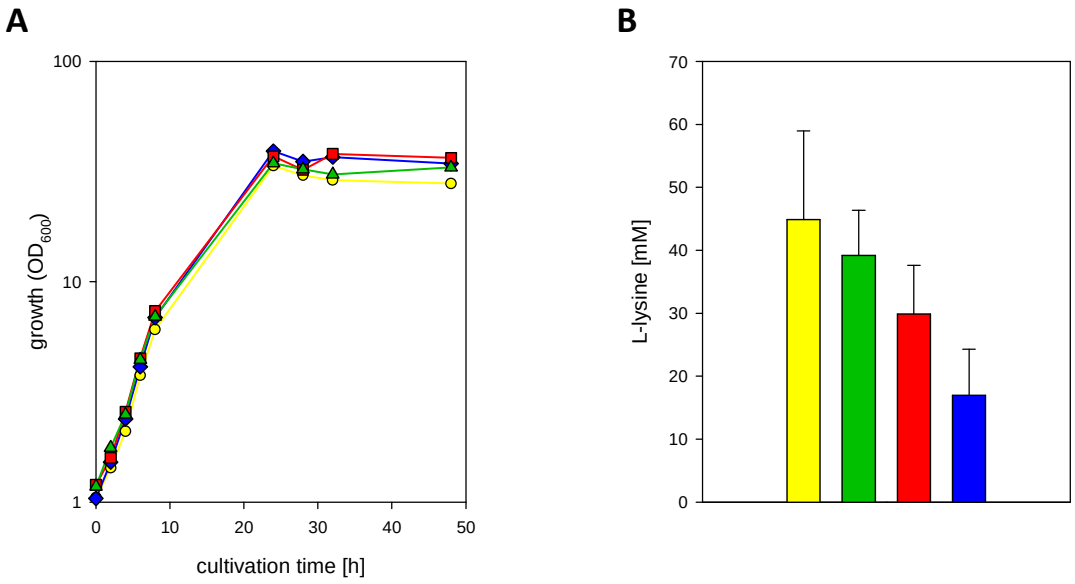

Figure 32: A: Growth of *C. glutamicum* DM1729 Δ*ilvE* ○, *C. glutamicum* DM1729 Δ*ilvE dapA*-Prom:B31 △, *C. glutamicum* DM1729 Δ*ilvE dapA*-Prom:C13 □ and *C. glutamicum* DM1729 Δ*ilvE dapA*-Prom:C5 ◇ in CgXII minimal medium + 4 % glucose + L-valine, L-leucine and L-isoleucine (2mM each). **B:** L-lysine in the culture supernatants of the experiments shown in A after 24 hours. Same color code as in A.

4. Discussion

The growth experiments with *C. glutamicum* in the presence of KMV showed that this α-keto acid has a strong inhibiting effect on the growth. The OD_{600} stagnates between 4 and 5 for at least 30 hours. A similar effect was also observed with KIV and KIC, even though in a lesser extent. Experiments in which the growth could be restored by the addition of yeast extract or the three branched-chain amino acids L-valine, L-leucine and L-isoleucine give the hint that KMV negatively influences the synthesis of these amino acids. This may either happen due to a competitive inhibition of the transaminase B by such an unnaturally high KMV concentration so that KIV and KIC are no longer converted to the corresponding amino acids in a sufficient amount. Or KMV inhibits one or several of the commonly used enzymes of the KIV and KMV synthesis, which there are AHAS, AHAIR and DHAD. The first possibility is largely debilitated by the fact that it is also possible to restore growth of a KMV-containing culture by the single addition of KIV.

The pyruvate plus pyruvate reaction of the AHAS was shown to be competitively inhibited by high concentrations of KMV. Additionally the enzyme is allosterically inhibited by isoleucine and the expression of its genes is also repressed by isoleucine that is formed from the KMV. All these aspects may result in an AHAS activity to low for a sufficient synthesis of valine and leucine. On the other hand the 2-oxobutyrate plus pyruvate reaction of the AHAS is not affected by KIV but only by valine which may explain the growth in the presence of KIV. Although the pyruvate plus pyruvate reaction is inhibited by KIV even stronger (Krause, 2010) this does not affect growth, because both valine and leucine can be synthesized from KIV. For a better understanding of the whole AHAS regulation, the AHAS activity should also be characterized in the presence of KIC and leucine. In cultures containing KIC a growth inhibition was observed, even though less severe than with KMV. If KIC inhibits the AHAS this would also result in a deprivation of L-valine. Thus, the effect of KIC on the AHAS is possibly weaker than that of KMV so that L-valine synthesis is still possible and *C. glutamicum* is able to grow slowly in the presence of KIC.

The growth experiments with *C. glutamicum* in the presence of branched-chain α-keto acids revealed that their concentrations decrease during growth. By incubating the branched-chain α-keto acids in cell-free medium it was analyzed if the molecules are chemically stable under the present conditions. It was shown in this work and also by Krause (2010) that there is no chemical degradation of branched-chain α-keto acids in fresh cell-free medium, so the concentration decrease is due to the presence of bacteria. *C. glutamicum* is able to take up branched-chain α-keto acids; however a specific transport protein has not yet been identified

(Radespiel, 2010). As shown in the growth experiments the degradation of branched-chain α-keto acids did not result in a higher OD_{600}. Thus, *C. glutamicum* is not able to use KIV, KIC and KMV as carbon sources for biomass formation. Additionally, *C. glutamicum* was not able to grow with KMV as the only carbon source. For the wild type strain it was shown that a bioconversion of KMV to L-isoleucine takes place, but also with the transaminase B-negative strain *C. glutamicum* Δ*ilvE* a degradation of branched-chain α-keto acids was observed. Therefore there has to be some different degradation mechanism next to the transamination to the correspondent amino acids, although no enzyme system for the degradation of branched-chain α-keto acids has been described in *C. glutamicum*. A possible candidate would be a BKDHC, which oxidatively decarboxylates the branched-chain α-keto acids with simultaneous reduction of NAD^+ and the formation of a CoA-adduct of the substrate. Such an enzyme complex has been described for several different bacterial species with diverse metabolic roles: In *Pseudomonas putida* and *E. faecalis* it is part of the catabolic degradation of branched-chain amino acids in order to gain ATP via substrate level phosphorylation (Sokatch *et al.*, 1981; Ward *et al.*, 2000), in *B. subtilis* the BKDHC is necessary for the synthesis of branched-chain fatty acids that are part of the membrane composition (Wang *et al.*, 1993; Lowe *et al.*, 1983). However, since *C. glutamicum* is neither able to catabolize branched-chain amino acids nor does it contain branched-chain fatty acids (Collins *et al.*, 1982), the existence of a BKDHC in *C. glutamicum* is rather unlikely. On the other hand it has been shown that the PDHC may catalyze unspecific reactions such as the conversion of 2-oxobutyrate by the PDHC of *Salmonella typhimurium* (van Dyk *et al.*, 1987). Therefore experiments were made with *lpd*- and *odhA*-negative strains. However those strains showed no different behavior in their ability to degrade branched-chain α-keto acids in comparison to the wild type of *C. glutamicum*. Furthermore no accordant enzyme activity could be detected, when branched-chain α-keto acids were used as substrate even in high concentrations. Thus, the degradation of KIV, KIC and KMV probably is neither caused by a BKDHC nor by unspecific reactions of the PDHC or ODHC in *C. glutamicum*.

Another enzyme that converts branched-chain α-keto acids is the 2-hydroxy acid dehydrogenase of *L. lactis* which reduces them to 2-hydroxy acids with simultaneous oxidation of NADH (Chambellon *et al.*, 2009). In this organism it probably has the physiological role to regenerate NAD^+ when branched-chain amino acids are catabolized. However by performing the enzyme test, no evidence could be found for the existence of a 2-hydroxy acid dehydrogenase in *C. glutamicum*. Further enzymes that convert branched-chain α-keto acids are non-oxidative keto acid decarboxylases which catalyze their decarboxylation to aldehydes as part of the Ehrlich pathway for the degradation of amino acids. This pathway has extensively been analyzed in *Saccharomyces cerevisiae* (Hazelwood *et al.*, 2008), but non-oxidative keto acid decarboxylases have also been identified in the bacterial species *L. lactis* (Smit *et al.*, 2005) and *M. tuberculosis*

(Werther et al., 2007). However, in contrast to these organisms, *C. glutamicum* is not able to catabolize branched-chain amino acids as carbon and energy source and also sequence homology analysis (data not shown) did not yield an evidence for such an enzyme in *C. glutamicum*. So the most probable explanation is that there is no specific branched-chain α-keto acid degrading enzyme in *C. glutamicum*, but the degradation is caused by unspecific reactions of other α-keto acid converting enzymes. In a work focusing on the production of isoleucine from 2-hydroxybutyrate several byproducts were identified and possible unspecific reactions of the AHAS and the isopropylmalate synthase are described (Wilhelm *et al.*, 1989). For the latter enzyme it has been shown in *E. coli* as well as in *Serratia marcescens* that it can use alternative substrates quite efficiently, if they are present in high concentrations (Sycheva *et al.*, 2007; Kisumi *et al.*, 1976). The enzymes of the pantothenate synthesis are also known to transform KMV as well as 2-oxobutyrate and 2-oxovalerate next to their substrate KIV, each leading to a different product (Powers *et al.*, 1976). Further candidates for unspecific branched-chain α-keto acid degrading enzymes could be pyruvate converting enzymes. Krause (2010) has investigated the degradation of KIV by *C. glutamicum* strains deficient for the lactate dehydrogenase or the pyruvate:quinine oxidoreductase. With the lactate dehydrogenase-negative strain a slightly reduced KIV degradation was observed. For the pyruvate:quinine oxidoreductase it is known that the enzyme is able to convert KIV, KIC and KMV with a low specific activity (Schreiner, 2004), however Krause could not find any evidence that a deletion of the *pqo*-gene has an influence on KIV degradation.

Further experiments have shown that degradation of branched-chain α-keto acids does not only take place in the presence of *C. glutamicum* cells but also in sterile filtrated culture supernatant even though in a lesser extent. In contrast to fresh medium KIV, KIC and KMV were not stable in such “spent” media. An enzyme-catalyzed degradation of the branched-chain α-keto acids can almost be excluded in this case, since the heating of the culture supernatant did not have an effect on the speed of the concentration decrease. At this temperature a complete denaturation of *C. glutamicum* proteins would have been expected and thus the concentration decrease in “spent” medium was most likely caused by chemical degradation. Since a concentration decrease was not observed in fresh medium, a reactive metabolite which interacts with the branched-chain α-keto acids has to be produced by growing *C. glutamicum* cells.

The activity assays with the AHAS in order to characterize this key enzyme in the presence of branched-chain α-keto acids showed that the pyruvate + pyruvate reaction of the AHAS is inhibited by high concentrations of KMV. Before, it has been shown by Krause (2010) that KIV inhibits this reaction of the AHAS. Since the AHAS is allosterically regulated by the branched-chain amino acids it could be assumed that the inhibition is caused by an interaction of KMV

with the regulatory subunit of the AHAS due to its structural similarity to L-isoleucine. However the characteristics of the inhibition with a threefold increase of the K_m-value for pyruvate and an almost unaffected maximum enzyme activity are typical features of a competitive inhibition. The 2-oxobutyrate + pyruvate reaction in contrast remained unaffected by high KMV concentrations. This may be due to the higher affinity of the AHAS towards 2-oxobutyrate in comparison to pyruvate. In further experiments the 2-oxobutyrate + pyruvate reaction of the AHAS was also analyzed with regard to an inhibition by L-isoleucine and L-valine. As it has been observed with the pyruvate + pyruvate reaction (Blombach *et al.*, 2009), a 30 to 40 % lower specific activity was detected in the presence of L-valine or L-isoleucine (10 mM each). The K_m-value for 2-oxobutyrate however remained on the same level, or, most remarkably in the case of L-valine, was more than 50 % lower. A reduced K_m-value in the presence of L-valine has also been described for the TD (Möckel *et al.*, 1992). The regulation of the TD helps balancing the flux through the competing pathways of L-valine and L-isoleucine biosynthesis which would also be an appropriate explanation for a lower K_m-value of the AHAS for 2-oxobutyrate in the presence of L-valine. The differences between the inhibition of the AHAS caused by KMV and the inhibiting effect of branched-chain amino acids strengthen the assumption also stated by Krause *et al.* (2010) that two totally separate inhibition mechanisms are present. While an allosteric inhibition takes place when branched-chain amino acids interact with the regulatory subunit of the AHAS, branched-chain α-keto acids cause a competitive inhibition by interaction with the substrate binding site of the catalytic subunit. With regard to the construction of a KMV production strain, the results obtained for the AHAS were quite positive as the 2-oxobutyrate + pyruvate reaction is not inhibited by KMV even in high concentrations.

As a further key enzyme of KMV synthesis the anabolic TD of *C. glutamicum* was also analyzed in its activity in the presence of KMV. As the only unique enzyme of L-isoleucine biosynthesis it is severely inhibited by L-isoleucine (Möckel *et al.*, 1992). A regulatory effect of KMV could not be excluded due to the structural similarity towards L-isoleucine. The enzyme assays showed an inhibition of about 50 % in the presence of high KMV concentrations (50 mM). However this inhibition could be compensated by doubling the concentration of the substrate L-threonine which indicates competitive inhibition by KMV and not an allosteric regulation. The feedback resistant variants of the TD showed the same inhibition by KMV as the native enzyme which strengthens the assumption that the inhibition by L-isoleucine and the inhibition by KMV have a different mechanism. This result could be validated by a closer characterization of the TD with the determination of the K_m-value in the comparison with and without KMV. The TD already has a high K_m-value of about 20 mM L-threonine (Möckel *et al.*, 1992) which is possibly increased by KMV. Thus, with regard to the construction of a KMV producer the experiments made obvious that a high intracellular L-threonine concentration is necessary for an efficient KMV synthesis.

The first experiments to evaluate if *C. glutamicum* is able to produce KMV were performed by feeding different strains of *C. glutamicum* with 2-oxobutyrate as second substrate next to glucose. Those experiments proved that the biotechnological synthesis of KMV with *C. glutamicum* is possible. More than 70 % of the supplied 2-oxobutyrate was converted to KMV. The experiment showed that the synthesis pathway downstream of 2-oxobutyrate including the enzymes AHAS, AHAIR and DHAD is open for KMV production when enough substrate is present. Consistently, the overexpression of the respective genes *ilvBNCD* did not result in a faster KMV formation. The same effect was observed in a work dealing with the production of L-isoleucine from 2-oxobutyrate (Eggeling *et al.*, 1987). Here a more than 10-fold higher specific AHAS activity was detected in the presence of 2-oxobutyrate. However, the mechanism of this regulation is not known as no transcriptional regulator of the *ilvBNC* operon has been described so far. A deprivation of L-valine and L-leucine caused by the extremely strong flux towards L-isoleucine which has formerly been used as an explanation (Eggeling *et al.*, 1987; Keilhauer *et al.*, 1993) is rather unlikely as only reason in the present case as all three branched-chain amino acids have been supplemented in cultures of the *ilvE*-deleted strains. The supplementation may also be the reason why the addition of 2-oxobutyrate did not cause a long lag phase as observed by Eggeling *et al.* (1987).

With the results of the fermentation experiments with 2-oxobutyrate as substrate in mind, a KMV production strain on the basis of the L-lysine producer *C. glutamicum* DM1729 was constructed aiming on a high intracellular 2-oxobutyrate concentration. One remarkable effect that was observed was the about 60 % higher L-lysine excretion of *C. glutamicum* DM1729 Δ*ilvE* compared to *C. glutamicum* DM1729. The results suggest that there is a link between the L-lysine biosynthesis and the synthesis of branched-chain amino acids. A similar effect was observed by Blombach *et al*. (2009) when *ilvB*, coding for the catalytic subunit of the AHAS, was deleted in *C. glutamicum* DM1729. Same as *C. glutamicum* DM1729 Δ*ilvE*, *C. glutamicum* DM1729 Δ*ilvB* is auxotrophic for the branched-chain amino acids. The authors made a comparative transcriptome analysis between *C. glutamicum* DM1729 and *C. glutamicum* DM1729 Δ*ilvB* and found more than forty genes with altered mRNA levels. However a direct influence on the transcription of the L-lysine synthesis genes was not detected. In another study *leuC*, coding for the 3-isopropylmalate dehydratase was mutated in *C. glutamicum* DM1729 which lead to a partial L-leucine auxotrophy (Hayashi *et al.*, 2006). In this strain also an increased L-lysine excretion was observed. In the *leuC*-mutant the *lysC-asd* operon (encoding aspartate kinase and aspartate semialdehyde dehydrogenase) was upregulated together with several other amino acid biosynthesis genes. Thus, the authors proposed the hypothesis that there may be a global regulation for amino acid biosynthesis. Furthermore some L-lysine production strains derived by classic mutagenesis are also L-leucine auxotrophs (Tosaka *et al.*, 1978), such as *C. glutamicum*

B-6 (Hirao *et al.*, 1989). Thus, leucine auxotrophy seems to be beneficial for lysine biosynthesis. Thus, it is possible that the increased L-lysine production of *C. glutamicum* DM1729 Δ*ilvE* is caused by the L-leucine auxothrophy (and/or L-isoleucine auxotrophy), although the regulatory mechanism is not known.

C. glutamicum DM1729 Δ*ilvE* was subsequently transformed with plasmids harboring the threonine biosynthesis genes and *ilvA* in order to redirect the carbon flux away from L-lysine synthesis but to KMV synthesis. With the final KMV production strain *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) for the first time the intentional synthesis of KMV from glucose as only carbon source was achieved, even though the final KMV concentration was still low. The drastically reduced excretion of L-lysine showed that by overexpression of the threonine synthesis genes plus *ilvA* the flux could be driven away from L-lysine synthesis. On the other hand the difference between the decrease of L-lysine excretion and the increase in KMV production made obvious that there had to be a bottleneck in the KMV synthesis pathway. This bottleneck was found out to be caused by the supplemented branched-chain amino acids, since *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) produced up to 35 mM KMV without any supplementation. Most surprisingly the strain was able to grow under these conditions. The transaminase B whose gene was deleted in the production strain is described to be the only transaminase responsible for the synthesis of L-isoleucine and L-leucine (Marienhagen *et al.*, 2005). A possible explanation for this effect could be that a high intracellular concentration of the precursor KMV in the production strain allows a transamination of KMV to L-isoleucine unspecifically catalyzed by some other transaminase. A likely candidate would be the valine-pyruvate transaminase (encoded by *avtA*) which aminates KIV to L-valine with L-alanine as amino group donor (Marienhagen *et al.*, 2008).

The impact of the branched-chain amino acids on growth behavior and product formation was analyzed more in detail by the separate addition of L-isoleucine, L-valine and L-leucine in growth experiments. Hereby it was observed that especially L-isoleucine has a strong inhibiting impact on KMV synthesis, but on the other hand it allows a much higher growth rate. L-valine in contrast severely inhibited the growth while the impact of L-leucine was rather weak and possibly slightly positive for KMV synthesis. The combined addition of the branched-chain amino acids showed that the effect of L-isoleucine is dominant over the other two amino acids. As the most likely target of L-isoleucine the TD was identified that is known to be strictly inhibited by this amino acid (Möckel *et al.*, 1992). The dramatic effect of L-valine on the growth is more difficult to explain: None of the enzymes specific for the synthesis of L-threonine and L-isoleucine are known to be inhibited by L-valine; only the AHAS is both repressed in its expression and inhibited in its activity by L-valine (Morbach *et al.*, 2000, Blombach *et al.*, 2009).

Measurements of the respective enzyme activity showed a reduced AHAS activity in the presence of L-valine in comparison to *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) grown without L-valine, but still a high specific activity if compared to the wild type of *C. glutamicum*. However the reduced AHAS activity may result in a lower intracellular concentration of KMV and KIC which led to a deprivation of L-isoleucine and L-leucine. As a consequence of these experiments the gene for the native TD (*ilvA*) was exchanged against a version coding for a feedback resistant enzyme (*ilvA* D378G; Möckel *et al.*, 1994) to avoid an inhibition of KMV production by L-isoleucine. However *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pECM3 *ilvA* D378G) did not perform significantly different than *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) both in the presence and absence of L-isoleucine. By measuring the specific TD activity it turned out that IlvA D378G was resistant against L-isoleucine in concentrations up to 1 mM, a concentration at which the wild type enzyme was already inhibited by more than 80 %. Möckel *et al.* (1994) selected their feedback-resistant enzymes at an isoleucine concentration of 0.625 mM. With the 2 mM L-isoleucine present as supplement in growth experiments however, the feedback resistant TD did not possess a significant advantage and therefore no higher KMV production could be expected by expressing *ilvA* D378G.

A second approach with a feedback resistant TD was the expression of the catabolic TD from *E. coli* (*tdcB*) in *C. glutamicum* which has already successfully been used with a L-isoleucine production strain (Guillouet *et al.*, 1999). TdcB possesses a very high resistance against L-isoleucine and also a high tolerance against KMV was observed. These characteristics of the catabolic TD made it a promising alternative to the anabolic TD of *C. glutamicum*. Subsequently, "pMM36ptac *tdcB*" was transformed into the KMV production strain instead of "pMM36ptac *ilvA*". The resulting strain *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *tdcB*) however did not fulfill the expectations as the produced KMV concentration was lower than before while more L-lysine was excreted. Activity measurements revealed a very low specific TD activity in the current strain. Together with the observation that "pMM36ptac *tdcB*" yielded very weak bands in plasmid preparations made as controls (data not shown), the results hint on a suppression of the plasmid in the KMV production strain. Possibly "pMM36ptac *tdcB*" is only present in a low copy number if applied as second plasmid next to "pEK *hom*^{FBR} *thrB thrC*". A disadvantage of the use of TdcB is its dependence on AMP as a cofactor (Hirata *et al.*, 1965) which was also observed in the current work. In its active complementation the enzyme forms a tetramer which is only stable in the presence of AMP. In the absence of AMP TdcB dissociates in single subunits or dimers which do not possess the full enzymatic activity (Whanger *et al.*, 1968). In *E. coli tdcB* is only expressed in the absence of glucose when the organism suffers from energy deprivation and AMP is present in a high concentration (Umbarger and Brown, 1957). In

the KMV production strain however which is grown with sufficient carbon supply the adenine nucleotides are mostly present as ATP, the AMP concentration may be too low for the catabolic TD to form stable tetramers which would result in a reduced specific enzyme activity.

Fed batch fermentations of *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) with glucose and 2-oxobutyrate as substrates showed that higher concentrations of KMV might be produced. As a maximal concentration 150 mM KMV were produced from a total amount of 200 mM 2-oxobutyrate and 300 mM glucose. However both biomass formation and KMV formation ceased when this concentration was reached, although there was still substrate present in the medium. A depletion of some other medium component may be an explanation, but on the other hand the excretion of L-lysine and L-threonine was increased when the strain ceased to produce further KMV. Thus, it is more likely that the high KMV concentration has an inhibiting impact on any of the enzymes responsible for the synthesis of branched-chain amino acids and also KMV. That would also be an explanation for the stop of further growth, caused by a lack of the branched-chain amino acids L-valine and L-leucine. It has to be evaluated more closely if 150 mM are a limiting concentration for the production of KMV.

In another experiment *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) was grown with glucose and L-homoserine as carbon sources. The amount of produced KMV did only slightly increase in comparison to fermentations with glucose as only carbon source, although L-homoserine was largely taken up by the bacteria during growth. The concentration of L-lysine however was much higher as well as the excreted L-threonine. Thus, it can be assumed that the measured KMV is derived from the supplemented L-homoserine, while the carbon flux from glucose was mostly channeled into L-lysine. The high concentration of L-homoserine may have somehow prevented the flux of aspartate semialdehyde to the threonine branch, probably by inhibiting one or several of the enzymes. Especially the HOM would be a candidate as it is the first enzyme of the threonine biosynthesis branch. The HOM is known to be inhibited by L-threonine (Eikmanns *et al.*, 1991; Myajima et al., 1968), but there are no studies available on its activity in the presence of high concentrations of its own product, homoserine. The excretion of L-threonine is always a hint on an inhibition of the TD so it should also be investigated if the TD is somehow influenced by high concentrations of homoserine.

The experiments performed in this work showed that it is rather unlikely that *C. glutamicum* possesses enzymes specifically degrading branched-chain α-keto acids. Thus, the observed degradation of KMV, KIV and KIC is probably caused by unspecific catalysis of several enzymes and at least partially by chemical degradation. Since their degradation may also be problematic for the biotechnological production of the branched-chain α-keto acids, a further investigation of

this topic is recommended. An approach could be to try to identify a degradation product and on this way to conclude on a possible enzyme.

In summary the present work could show that a biotechnological synthesis of KMV is possible with *C. glutamicum*. A strain has been constructed that produces up to 35 mM KMV from glucose as only carbon source depending on growth conditions. There are several possibilities for the improvement of the current KMV production strain: One would be to avoid the excretion of L-lysine as a byproduct because the lysine biosynthesis competes with the KMV synthesis for the common precursor aspartate semialdehyde. The work on this aspect has already begun with the exchange of the promoter of *dapA* (encoding the dihydrodipicolinat synthase) against weaker promoters (Vašicova *et al.*, 1999). Hereby the flux into L-lysine biosynthesis could be reduced what became visible as the concentration of excreted L-lysine was lower in consistence with the lower promoter activities. By overexpression of *hom*^{FBR}, *thrB*, *thrC* and *ilvA* the carbon flux should be redirected towards KMV synthesis and possibly a higher concentration could be achieved. Another aspect which can be improved is the expression of *ilvA* or especially a feedback resistant variant of *ilvA* or alternatively *tdcB*. The expression of those genes on a second plasmid turned out to be somehow problematic, since the measured enzyme activities were low compared to strains harboring only one plasmid. Because the expression of a TD gene together with *hom*^{FBR} on a single plasmid was not successful, a solution could be the genomic expression of a feedback resistant variant of *ilvA* by exchange of the native gene. This approach would also exclude the possibility of mixed enzyme complexes, since the TD forms a tetramer (Möckel *et al.*, 1992). Furthermore the threonine biosynthesis genes should be analyzed more closely, especially if they are inhibited by KMV and also by homoserine. The supplementation experiments with homoserine made it obvious that the pathway from aspartate semialdehyde to 2-oxobutyrate is not completely open. Special interest would lie on the HOM, since this enzyme catalyzes the first reaction of the threonine branch. Finally the experiments of the current work showed the strong influence of the medium composition on the behavior of bacterial cultures. The severe growth inhibition of KMV could almost completely be reversed by the addition of branched-chain amino acids or complex substances. And also with the KMV production strain alterations with the supplementary amino acids led to a strong increase of KMV production and also helped to identify enzymes of the KMV biosynthesis pathway that underlie an inhibition.

Literature

Abe S., K.-I. Takayama, and S. Kinoshita. 1967. Taxonomical studies on glutamic acid-producing bacteria. *J. Gen. Appl. Microbiol.* **13:** 279-301

Aparicio, M., N. J. M. Cano, A. Cupisti, T. Ecder, D. Fouque, L. Garneata, H. Liou, S. Lin, H. Schober-Halstenberg, V. Teplan, and G. Zakar. 2009. Keto-acid therapy in predialysis chronic kidney disease patients: concencus statements. *J. Ren. Nutr.* **19:** 33-35.

Bayan, N., C. Houssin, M. Chami, and G. Leblon. 2003. Mycomembrane and S-layer: two important structures of *Corynebacterium glutamicum* cell envelope with promising biotechnology applications. *J. Biotechnol.* **104:** 55-67.

Birnboim, H. C. 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol.* **100:** 243-255.

Blombach, B., M. E. Schreiner, M. Koch, M. Oldiges, and B. J. Eikmanns. 2007. Effect of pyruvate dehydrogenase complex deficiency on L-lysine production with *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **76:** 615-623.

Blombach, B., S. Hans, B. Bathe, and B. J. Eikmanns. 2009. Acetohydroxyacid synthase, a novel target for improvement of L-lysine production by *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* **75:** 419-427.

Bullock, W. O., J. M. Fernandez, and J. M. Short. 1987. XL1-Blue: A high efficiency plasmid transforming *rec*A *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* **5:** 376-379.

Chambellon, E., L. Rijnen, F. Lorquet, C. Gitton, J. E. T. van Hylckama Vlieg, J. O. Wouters, and M. Yvon. 2009. The D-2-hydroxyacid dehydrogenase incorrectly annotated PanE is the sole reduction system for branched-chain 2-keto acids in *Lactococcus lactis*. *J. Bacteriol.* **191:** 873-881.

Cohen, G. N., R. Y. Stanier, and G. le Bras. 1969. Regulation of the biosynthesis of amino acids of the aspartate family in coliform bacteria and Pseudomonads. *J. Bacteriol.* **99:** 791-801.

Collins, M. D., M. Goodfellow, and D. E. Minnikin. 1982. Fatty acid composition of some mycolic acid-containing coryneform bacteria. *J. Gen. Microbiol.* **128:** 2503-2509.

Cooper, A. J. L., J. Z. Ginos and A. Meister. 1983. Synthesis and properties of the α-keto acids. *Chem. Rev.* **83:** 321-358.

Creaghan, I. T., and J. R. Guest. 1972. *Amber* mutants of the α-ketoglutarate dehydrogenase gene of *Escherichia coli* K12. *J. Gen. Microbiol.* **71:** 207-220.

Cremer, J., C. Treptow, L. Eggeling, and H. Sahm. 1988. Regulation of lysine biosynthesis in *Corynebacterium glutamicum*. *J. Gen. Microbiol.* **134:** 3221-3229.

Cremer, J., L. Eggeling, and H. Sahm. 1991. Control of the lysine biosynthesis sequence in *Corynebacterium glutamicum* as analyzed by overexpression of the individual corresponding genes. *Appl. Environ. Microbiol.* **57:** 1746-1752.

Daffé, M. 2005. The cell envelope of *Corynebacteria*. In: Handbook of *Corynebacterium glutamicum*, Eggeling, L., & M. Bott (Eds.), CRC Press, Boca Raton, FL., 121-148.

Eggeling, I., C. Cordes, L. Eggeling, and H. Sahm. 1987. Regulation of acetohydroxy acid synthase in *Corynebacterium glutamicum* during fermentation of α-ketobutyrate to L-isoleucine. *Appl. Microbiol. Biotechnol.* **25:** 346-351.

Eikmanns, B. J., M. Metzger, D. Rheinscheid, M. Kirchner, and H. Sahm. 1991. Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and ist influence on carbon flux in different strains. *Appl. Microbiol. Biotechnol.* **34:** 617-622.

Eikmanns, B. J., N. Thum-Schmitz, L. Eggeling, K. Lüdtke, and H. Sahm. 1994. Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. *Microbiology* **140:** 1817-1828.

Elisáková, V., M. Pátek, J. Holátko, J. Nesvera, D. Leyval, L. Goergen, and S. Delaunay. 2005. Feedback-resistant acetohydroxy acid synthase increases valine production in *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* **71:** 207-213.

Feiten, S. F., S. A. Draiba, R. Watanabe, M. R. Duenhas, A. C. Baxman, F. B. Nerbass, and L. Cuppari. 2005. Short-term effects of a very-low-protein diet supplemented with ketoacids in nondialyzed chronic kidney disease patients. *Eur. J. Clin. Nutr.* **59:** 129-136.

Follettie, M. T., H. K. Shin, and A. J. Sinskey. 1988. Organization and regulation of the *Corynebacterium glutamicum hom-thrB* and *thrC* loci. *Mol. Microbiol.* **2:** 53-62.

Frères, R., and F. R. Lestrem. 2001. Nährungszusammensetzung aus dem Maisquellwasser und Verfahren zu deren Herstellung. German patent DE69703371T2 03.05.2001.

Freundlich, M., R. O. Burns, and H. E. Umbarger. 1962. Controll of isoleucine, valine and leucine biosynthesis, I. multi-valent repression. *Proc. Natl. Acad. Sci. U S A*. **48:** 1804-1808.

Funke, G., A. von Graevenitz, J. E. Clarridge III, and K. A. Bernard. 1997. Clinical microbiology of coryneform bacteria. *Clin. Microbiol. Rev.* **10:** 125-159.

Georgi, T., D. Rittmann, and V. F. Wendisch. 2005. Lysine and glutamate production by *Corynebacterium glutamicum* on glucose, fructose and sucrose: Roles of malic enzyme and fructose-1,6-bisphosphatase. *Metab. Eng.* **7:** 291-301.

Guillouet, S., A. A. Rodal, G.-H. An, P. A. Lessard, and A. J. Sinskey. 1999. Expression of the *Escherichia coli* catabolic threonine dehydratase in *Corynebacterium glutamicum* and its effect on isoleucine production. *Appl. Environ. Microbiol.* **65:** 3100-3107.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166:** 557-580.

Hara, S., Y. Takemori, M. Yamaguchi, M. Nakamura, and Y. Ohkura. 1985. Determination of alpha-keto acids in serum and urine by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr.* **344:** 33-39.

Hayashi, M., H. Mizoguchi, J. Ohnishi, S. Mitsuhashi, Y. Yonetani, S. Hashimoto, and M. Ikeda. 2006. A *leuC* mutation leading to increased L-lysine production and *rel*-independent global expression changes in *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **72:** 783-789.

Hazelwood, L. A., J.-M. Daran, A. J. A. van Maris, J. T. Pronk, and J. R. Dickinson. 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl. Environ. Microbiol.* **74:** 2259-2266.

Hell, D. 2010. Versuche zur Verbesserung der 2-Keto-3-methylvalerat Produktion in *Corynebacterium glutamicum*. Bachelor thesis, made at the institute for microbiology and biotechnology at the University of Ulm.

Hermann, T. 2003. Industrial production of amino acids by coryneform bacteria. *J. Biotechnol.* **104:** 155-172.

Hirao, T., T. Nakano, T. Azuma, M. Sugimoto, and T. Nakanishi. 1989. L-lysine production in continuous culture of an L-lysine hyperproducing mutant of *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **32:** 269-273.

Hirata, M., M. Tokushige, A. Inagaki, and O. Hayaishi. 1965. Nucleotide activation of threonine deaminase from *Escherichia coli*. *J. Biol. Chem.* **240:** 1711-1717.

Hoffelder, M., K. Raasch, J. van Ooyen, and L. Eggeling. 2010. Pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase: The E2 domain of OdhA of *Corynebacterium glutamicum* has succinyltransferase activity dependent on lipoyl residues of the acetyltransferase AceF. *J. Bacteriol.* **192:** 5203-5211.

Ikeda, M. 2003. Amino acid production process. *Adv. Biochem. Eng. Biotechnol.* **79:** 1-35.

Ikeda, M., and S. Nakagawa. 2003. The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. *Appl. Microbiol. Biotechnol.* **62:** 99-109.

Kalinowski, J., B. Bathe, D. Barthels, M. Bischoff, M. Bott, A. Burkovski, N. Dusch, L. Eggeling, B. J. Eikmanns, L. Gaigalat, A. Goesmann, M. Hartmann, K. Huthmacher, R. Krämer, B. Linke, A. C. McHardy, F. Meyer, B. Möckel, W. Pfefferle, A. Pühler, D. A. Rey, C. Rückert, O. Rupp, H. Sahm, V. F. Wendisch, I. Wiegräbe, and A. Tauch. 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J. Biotechnol.* **104:** 5-25.

Karau, A., M. Kottenhan, K. Drauz, N. Windhab, and A. Grünert. 2008. Food supplement containing alpha-keto acids. WO 2008/122613 A2.

Kase, H., and K. Nakayama. 1972. Production of L-threonine by analog-resistant mutants. *Agric. Biol. Chem.* **36:** 1611-1621.

Keilhauer, C., L. Eggeling, and H. Sahm. 1993. Isoleucine synthesis in *Corynebacterium glutamicum*: Molecular analysis of the *ilvB-ilvN-ilvC* Operon. *J. Bacteriol.* **175:** 5595-5603.

Kelle, R., T. Herrmann, D. Weuster-Botz, L. Eggeling, R. Krämer, and C. Wandrey. 1996. Glucose-controlled L-isoleucine fed-batch production with recombinant strains of *Corynebacterium glutamicum*. *J. Biotechnol.* **50:** 123-136.

Kelle, R., T. Hermann, and B. Bathe. 2005. L-lysine production. In: Handbook of *Corynebacterium glutamicum*, Eggeling, L., & M. Bott (Eds.), CRC Press, Boca Raton, FL., 465-488.

Kiefer, P., E. Heinzle, O. Zelder, and C. Wittmann. 2004. Comparative metabolic flux analysis of lysine-producing *Corynebacterium glutamicum* cultured on glucose or fructose. *Appl. Environ. Microbiol.* **70:** 229-239.

Kimura, E. 2005. L-glutamate production. In: Handbook of *Corynebacterium glutamicum*, Eggeling, L., & M. Bott (Eds.), CRC Press, Boca Raton, FL., 439-463.

Kinoshita S., S. Udaka, and M. Shimono. 1957. Studies on the amino acid fermentation. Production of L-glutamic acid by various microorganisms. *J. Gen. Appl. Microbiol.* **3:** 193-205

Kinoshita S., N. Nakayama, and S. Kitada. 1958. L-lysine production using microbial auxotroph. *J. Gen. Apppl. Microbiol.* **2:** 128-129.

Kirchner, O., and A. Tauch. 2003. Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. *J. Biotechnol.* **104:** 287-299.

Kisumi, M., M. Sugiura, and I. Chibata. 1976. Biosynthesis of norvaline, norleucine, and homoisoleucine in *Serratia marcescens*. *J. Biochem.* **80:** 333-339.

Klaffl, S., and B. J. Eikmanns. 2010. Genetic and functional analysis of the soluble oxaloacetate decarboxylase from *Corynebacterium glutamicum*. *J. Bacteriol.* **192:** 2604-2612.

Krause, F. S., A. Henrich, B. Blombach, R. Krämer, B. J. Eikmanns, and G. M. Seibold. 2010. Increased glucose utilization in *Corynebacterium glutamicum* by use of maltose, and ist application for the improvement of L-valine productivity. *Appl. Environ. Microbiol.* **76:** 370-374.

Krause, F. S., B. Blombach, and B. J. Eikmanns. 2010. Metabolic engineering of Corynebacterium glutamicum for 2-ketoisovalerate production. *Appl. Environ. Microbiol.* **76:** 8053-8061.

Krause, F. S. 2010. Rationale Stammentwicklung für die Produktion von 2-Ketoisovalerat und 2-Ketoisocaproat mit *Corynebacterium glutamicum*. Dissertation, made at the institute for mikrobiology and biotechnology of the University of Ulm.

Leuchtenberger, W., K. Huthmacher, and K. Drauz. 2005. Biotechnological production of amino acids and derivatives: current status and prospects. *Appl. Microbiol. Biotechnol.* **69:** 1-8.

Leyval, D., D. Uy, S. Delaunay, J. L. Goergen, and J. M. Engasser. 2003. Characterisation of the enzyme activities in the valine biosynthetic pathway in a valine-producing strain of *Corynebacterium glutamicum*. *J. Biotechnol.* **104:** 241-252.

Ligget, R. W., and H. Koffler. 1948. Corn steep liquor in microbiology. *Bacteriol. Rev.* **12:** 297-311.

Lindroth, P., and K. Mopper. 1979. High performance liquid chromatographic determination of subpicomole amounts of amino acids by proculomn fluorescence derivatization with o-pthaldialdehyde. *Anal. Chem.* **51:** 1667-1674.

Liebl, W., A. Bayerl, B. Schein, U. Stillner, and K. H. Schleifer. 1989. High efficience elctroporation of intact *Corynebacterium glutamicum* cells. *FEMS Microbiol. Lett.* **53:** 299-303.

Liebl, W. 1991. The genus *Corynebacterium*-nonmedical. *The prokaryotes*. New York, USA: Springer, 1157-1171.

Liebl, W. 2005. *Corynebacterium* taxonomy. In. Handbook of *Corynebacterium glutamicum*, Eggeling, L., & M. Bott (Eds.), CRC Press, Boca Raton, FL., 9-34.

Lowe, P. N., J. A. Hodgson, and R. N. Perham. 1983. Dual role of a single multienzyme complex in the oxidative decarboxylation of pyruvate and branched-chain 2-oxo acids in *Bacillus subtilis*. *Biochem. J.* **215:** 133-140.

MacGee, J., and M. Doudoroff. 1954. A new phosphorylated intermediate in glucose oxidation. *J. Biol. Chem.* **210:** 617-626.

Marienhagen, J., N. Kennerknecht, H. Sahm, and L. Eggeling. 2005. Functional analysis o all aminotransferase proteins inferred from the genome sequence of *Corynebacterium glutamicum*. *J. Bacteriol.* **187:** 7639-7646.

Marienhagen, J., and L. Eggeling. 2008. Metabolic function of *Corynebacterium glutamicum* aminotransferases AlaT and AvtA and impact on L-valine production. *Appl. Environ. Microbiol.* **74:** 7457-7462.

May, M. E., and L. L. Brown. 1989. Instability of orthophthalaldehyde reagent for amino acid analysis. *Anal. Biochem.* **181:** 135-139.

Minnikin, D. E., M. Goddfellow, and M. D. Collins. 1978. Lipid composition in the classification and identification of coryneform and related taxa. *Coryneform Bacteria*. London, UK: Academic Press, 85-160.

Miyajima, R., S.-I. Otsuka, and I. Shiio. 1968. Regulation of aspartate family amino acid biosynthesis in *Brevibacterium flavum*. *J. Biochem.* **63:** 139-148.

Möckel, B., L. Eggeling, and H. Sahm. 1992. Functional and structural analysis of threonine dehydratase from *Corynebacterium glutamicum*. *J. Bacteriol.* **174:** 8065-8072.

Möckel, B., L. Eggeling, and H. Sahm. 1994. Threonine dehydratase of *Corynebacterium glutamicum* with altered allosteric control: their generation and biochemical and structural analysis. *Mol. Microbiol.* **13:** 833-842.

Morbach, S., H. Sahm, and L. Eggeling. 1996. L-isoleucine production with *Corynebacterium glutamicum*: Further flux increase and limitation of export. *Appl. Environ. Microbiol.* **62:** 4345-4351.

Morbach, S., C. Junger, H. Sahm, and L. Eggeling. 2000. Attenuation control of *ilvBNC* in *Corynebacterium glutamicum*: Evidence of leader peptide formation without the presence of a ribosome binding site. *J. Biosci. Bioeng.* **90:** 501-507.

Nunheimer T. D., J. Birnbaum, E. D. Ihnen, and A. L. Demain. 1970. Product inhibition of the fermentative formation of glutamic acid. *Appl. Microbiol.* **20:** 215-217.

Peters-Wendisch, P. G., B. J. Eikmanns, G. Thierbach, B. Bachmann, and H. Sahm. 1993. Phosphoenolpyruvate carboxylase in *Corynebacterium glutamicum* is dispensable for growth and lysine production. *FEMS Microbiol. Lett*. **112:** 269-274.

Peters-Wendisch, P. G., V. F. Wendisch, S. Paul, B. J. Eikmanns, and H. Sahm. 1997. Pyruvate carboxylase as an anaplerotic enzyme in *Corynebacterium glutamicum*. *Microbiology*. **143:** 1095-1103.

Powers, S. G., and E. E. Snell. 1976. Ketopantoate hydroxymethyltransferase. II. Physical, catalytic, and regulatory properties. *J. Biol. Chem.* **251:** 3786-3793.

Radespiel, T. 2010. Transport von organischen Säuren als Vorstufen der Aminosäurebiosynthese in *Corynebacterium glutamicum* und *Escherichia coli*. Dissertationsarbeit, angefertigt am Institut für Biochemie der Universität zu Köln.

Radmacher, E., A. Vaitsikova, U. Burger, K. Krumbach, H. Sahm, and L. Eggeling. 2002. Linking central metabolism with increased pathway flux: L-valine accumulation by *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* **68:** 2246-2250.

Reinscheid, D. J., B. J. Eikmanns, and H. Sahm. 1991. Analysis of a *Corynebacterium glutamicum hom* gene coding for a feedback-resistant homoserine dehydrogenase. *J. Bacteriol.* **173:** 3228-3230.

Rey, D. A., A. Pühler, and J. Kalinowski. 2003. The putative transcriptional repressor McbR, member of the TetR-family, is involved in the regulation of the metabolic network directing the synthesis of sulfur containing amino acids in *Corynebacterium glutamicum*. *J. Biotechnol.* **103:** 51-65.

Riedel, C., D. Rittmann, P. Dangel, B. Möckel, S. Petersen, H. Sahm, and B. J. Eikmanns. 2001. Characterization of the phosphoenolpyruvate carboxykinase gene from *Corynebacterium glutamicum* and significance of the enzyme for growth and amino acids production. *J. Mol. Microbiol. Biotechnol.* **3:** 573-583.

Sahm, H., and L. Eggeling. 1999. D-Pantothenate synthesis in *Corynebacterium glutamicum* and use of *panBC* and genes encoding L-valine synthesis for D-pantothenate overproduction. *Appl. Environ. Microbiol.* **65:** 1973-1979.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G.T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with the thermostable DNA polymerase. *Science (New York, N.Y.)* **239:** 487-491.

Sambrook, J., and D. W. Russel. 2001. *Molecular cloning*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory.

Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. *Gene* **145:** 69-73.

Schreiner, M. E. 2004. Physiologische und biochemische Untersuchungen zu den Pyruvat-decarboxylierenden Enzymen in Corynebacterium glutamicum. Dissertation, made at the institute for mikrobiology and biotechnology of the University of Ulm.

Shiio, I., S.-I. Otsuka, and M. Takahashi. 1962. Effect of biotin on the bacterial formation of glutamic acid. *J. Biochem.* **51:** 56-62.

Smit, B. A., J. E. T. van Hylckama Vlieg, W. J. M. Engels, L. Meijer, J. T. M. Wouters, and G. Smit. 2005. Identification, cloning, and characterization of a *Lactococcus lactis* branched-chain α-keto acid decarboxylase involved in flavor formation. *Appl. Environ. Microbiol.* **71:** 303-311.

Sokatch, J. R., V. McCully, and C. M. Roberts. 1981. Purification of a branched-chain keto acid dehydrogenase from *Pseudomonase putida*. *J. Bacteriol.* **148:** 647-652.

Stackebrandt, E., F. A. Rainey, and N. L. Ward-Rainey. 1997. Proposal for a new hierarchic classification system, *Actinobacteria* classic nov. *Int. J. Syst. Bacteriol.* **47:** 479-491.

Sycheva, E. V., T. A: Yampol'skaya, E. S. Preobrajenskaya, A. E. Novikova, N. G. Matrosov, and N. V. Stoynova. 2007. Overproduction of noncanonical amino acids by *Escherichia coli* cells. *Microbiology* **76:** 712-718.

Takeno S., J. Onishi, T. Komatsu, T. Masaki, K. Sen, and M. Ikeda. 2007. Anaerobic growth and potential for amino acid production by nitrate respiration in *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **75:** 1173-1182.

Teschan, P. E., G. J. Beck, J. T. Dwyer, T. Greene, S. Klahr, A. S. Levy, W. E. Mitch, L. G. Snetselaar, T. I. Steinman, and M. Walser. 1998. Effect of a ketoacid-aminoacid-supplemented very low protein diet on the progression of advanced renal disease: a reanalysis of the MDRD feasibility study. *Clin. Nephrol.* **50:** 273-28.

Tosaka, O., H. Hirakawa, K. Takinami, and Y. Hirose. 1978. Regulation of lysine biosynthesis by leucine in *Brevibacterium lactofermentum*. *Agric. Biol. Chem.* **42:** 1501-1506.

Umbarger, E., and B. Brown. 1957. Threonine deamination in *Escherichia coli*. II. Evidence for two L-threonine deaminases. *J. Bacteriol.* **73:** 105-112.

Umbarger, H. E. 1978. Amino acid biosynthesis and its regulation. *Ann. Rev. Biochem.* **47:** 533-606.

Van Dyk, T. K., and R. A. LaRossa. 1987. Involvement of *ack-pta* operon products in α-ketobutyrate metabolism by *Salmonella typhimurium*. *Mol. Gen. Genet.* **207:** 435-440.

Vašikova, P. M. Pátek, J. Nešvera, H. Sahm, and B. Eikmanns. 1999. Analysis of the *Corynebacterium glutamicum dapA* promoter. *J. Bacteriol.* **181:** 6188-6191.

Vertès, A. A., I. Masayuki, and H. Yukawa. 2005. Manipulating *Corynebacteria*, from individual genes to chromosomes. *Appl. Environ. Microbiol.* **71:** 7633-7642.

Wang, G., T. Kuriki, K. L. Roy, and T. Kaneda. 1993. The primary structure of branched-chain α-oxo acid dehydrogenase from *Bacillus subtilis* and its similarity to other α-oxo acid dehydrogenases. *Eur. J. Biochem.* **213:** 1091-1099.

Ward, D. E., C. C. van der Weijden, M. J. van der Merwe, H. V. Westerhoff, A. Claiborne, and J. L. Snoep. 2000. Brnached-chain α-keto acid catabolism via the gene products of the *bkd* operon in *Enterococccus faecalis*: a new, secreted metabolite serving as temporary redox sink. *J. Bacteriol.* **182:** 3239-3246.

Werther, T., M. Spinka, K. Tittmann, A. Schütz, R. Golbik, C. Mrestani-Klaus, G. Hübner, and S. König. 2008. Amino acids allosterically regulate the thiamine diphosphate-dependent α-keto acid decarboxylase from *Mycobacterium tuberculosis*. *J. Biol. Chem.* **283:** 5344-5254.

Whanger, P. D., A. T. Phillips, K. W. Rabinowitz, J. R. Piperno, J. D. Shada, and W. A. Woo. 1968. The mechanism of action of 5'-adenylic acid-activated threonine dehydrase. *J. Biol. Chem.* **243:** 167-173.

Wilhelm, C., I. Eggeling, A. Nassenstein, C. Jebsen, L. Eggeling, and H. Sahm. 1989. Limitations during hydroxybutyrate conversion to isoleucine with *Corynebacterium glutamicum*, as analyzed by the formation of by-products. *Appl. Microbiol. Biotechnol.* **31:** 458-462.

Wormser, E. H., and A. B. Pardee. 1958. Regulation of threonine biosynthesis in *Escherichia coli*. *Ach. Biochem. Biophys.* **78:** 416-432.

Yugari, Y. and C. Gilvarg. 1962. Coordinated end-product inhibition in lysine synthesis in *Escherichia coli*. *Biochim. Biophys. Acta.* **62:** 612-614.

Abbreviations

AHAIR	acetohydroxy acid isomeroreductase
AHAS	acetohydroxy acid synthase
AK	aspartate kinase
ASD	aspartate semialdehyde dehydrogenase
ATA	aspartate aminotransferase
ATP	adenosine-5'-triphosphate
BHI	brain heart infusion
BKDHC	branched-chain α-keto acid dehydrogenase complex
bp	base pairs
°C	degrees of Celcius
CoA	coenzyme A
CSL	corn steep liquor
DDB	1,2-diamino-4,5-dimethoxybenzol
DHAD	dihydroxy acid dehydratase
DNA	desoxyribunucleic acid
dNTP	desoxynucleotide-5'-triphosphate
ε	molar extinction coefficient
EDTA	ethylene diamin tetraacetic acid
et al.	et alii (and others)
F	farad (electric capacitance)
g	gram
GAP	glycerol aldehyde-3-phosphate

h	hour
HK	homoserine kinase
HOM	homoserine dehydrogenase
HPLC	high performance liquid chromatography
KIC	α-ketoisocaproate
KIV	α-ketoisovalerate
K_m	Michaelis-Menthen constant
KMV	α-ketomethylvalerate
l	liter
m	meter
M	molar (mol/l)
min	minute
mio	million
N	normal (mol equivalents per liter)
NAD(P)H + H^+	nicotinamidadenin dinucleotide (-2'-phosphate)
Ω	Ohm (electric resistance)
OD_{600}	optical density at 600 nm wavelength
ODHC	2-oxoglutarate dehydrogenase complex
OPA	ortho-phtaldialdehyde
PCR	polymerase chain reaction
PDHC	pyruvate dehydrogenase complex
PEP	phosphoenol pyruvate
pH	negative decade logarithm of the proton concentration
PPP	pentose phosphate pathway
PTS	phosphotransferase system

RP	reversed phase
rpm	rounds per minute
SDS	sodium dodecyl sulfate
sec	seconds
TA-B	transaminase B
TCA-cycle	tricarboxylic acid cycle
TD	threonine dehydratase
Tris	Tris-(hydroxymethyl-) aminomethane
U	unit (enzyme activity (µmol substrate/min))
UV	ultra violet
V	volt (electric voltage)
v/v	volume per volume
WT	wild type
w/v	weight per volume

Zusammenfassung

Die vorliegende Arbeit behandelt die biotechnologische Herstellung von α-Ketomethylvalerat (KMV) mit *Corynebacterium glutamicum*. Zusammen mit den anderen verzweigtkettigen α-Ketosäuren α-Ketoisovalerat (KIV) und α-Ketoisocaproat (KIC) wird KMV als pharmazeutischer Wirkstoff verwendet und kann auch als Inhaltsstoff von sogenanntem „Functional Food" eingesetzt werden. Bis jetzt wurde KMV ausschließlich über chemische Synthese hergestellt. Das Ziel der Arbeit war daher, die Entwicklung einer fermentativen KMV Herstellung einzuleiten.

Zunächst wurde der Einfluss von KMV, KIV und KIC auf den Stoffwechsel und das Wachstumsverhalten von *C. glutamicum* untersucht. Bei Versuchen, in denen *C. glutamicum* in Gegenwart der verzweigtkettigen α-Ketosäuren kultiviert wurde, stellte sich heraus, dass diese das Wachstum hemmen, insbesondere KMV. Bereits 50 mM KMV führten zu einer beinahe vollständigen Wachstumshemmung. Die Wachstumshemmung konnte durch die Zufütterung von L-Isoleucin, L-Valin und L-Leucin und auch durch Zugabe von KIC und KIC wieder aufgehoben werden. Dies deutet darauf hin, dass sich hohe Konzentrationen von KMV auf die Synthese der verzweigtkettigen Aminosäuren auswirken und das eingeschränkte Wachstum durch einen Mangel dieser Aminosäuren verursacht wird. Die Versuche offenbarten zudem, dass die verzweigtkettigen α-Ketosäuren während des Wachstums von *C. glutamicum* abgebaut werden. Der Wildtypstamm setzt diese effizient über Transaminierung zu den korrespondierenden verzweigtkettigen Aminosäuren um, doch auch in Kulturen des Transaminase B-negativen Stamms *C. glutamicum* Δ*ilvE* wurde eine signifikante Konzentrationsabnahme von KMV, KIV und KIC beobachtet. Es konnten in *C. glutamicum* keine Enzyme identifiziert werden, welche die verzweigtkettigen α-Ketosäuren spezifisch abbauen. Die wahrscheinlichste Erklärung ist daher, dass ein unspezifischer Abbau stattfindet, welcher durch andere, α-Ketosäuren-umsetzende Enzyme katalysiert wird.

Untersuchungen bei Schlüsselenzymen der KMV-Biosynthese zeigten, dass die Pyruvat-kondensierende Reaktion der Acetylhydroxysäure Synthase (AHAS) durch 100 mM KMV kompetitiv gehemmt wurde. Ein positives Ergebnis für die KMV-Herstellung war, dass sich die Kondensationsreaktion von 2-Oxobutyrat und Pyruvat desselben Enzyms als resistent gegenüber KMV erwiesen hat. Außerdem wurde gezeigt, dass die Kondensationsreaktion von 2-Oxobutyrat und Pyruvat der AHAS gleichermaßen von den verzweigtkettigen Aminosäuren inhibiert wird, wie die Pyruvat-kondensierende Reaktion. Bemerkenswert war hierbei der deutlich reduzierte K_m-Wert für 2-Oxobutyrat von etwa 6 mM auf unter 2.5 mM in Gegenwart von L-Valin. Ein solcher Regulationsmechanismus könnte dazu beitragen, dass auch in

Gegenwart hoher Konzentrationen von L-Valin die Versorgung mit L-Isoleucin sichergestellt ist. Auch die Threonindehydratase, für die eine starke allosterische Hemmung durch L-Isoleucin beschrieben ist, wurde durch KMV leicht gehemmt, vermutlich ebenfalls kompetitiv.

KMV wurde zunächst mit *C. glutamicum* Δ*ilvE* aus Glucose und 2-Oxobutyrat als zweitem Substrat hergestellt. Hierbei wurde eine effiziente Umsetzung von mehr als 70 % des eingesetzten 2-Oxobutyrats zu KMV erreicht. Dieses Ergebnis zeigte, dass der Stoffwechselweg ab 2-Oxobutyrat frei ist für die Synthese von KMV, was bei der Stammkonstruktion berücksichtigt wurde. Als Ausgangstamm für die Entwicklung eines KMV-Produktionsstamms wurde der Lysinproduzent *C. glutamicum* DM1729 verwendet. Die Pyruvatcarboxylase und die Aspartatkinase von *C. glutamicum* DM1729 sind dereguliert, was zur Bildung von Aspartatsemialdehyd führt, einem gemeinsamen Intermediat der L-Lysin- und der KMV-Biosynthese. In diesem Stamm wurde das *ilvE*-Gen, welches für die Transaminase B kodiert, deletiert, was zu einer um etwa 65 % gesteigerten L-Lysinausscheidung führte. Um den Kohlenstoffffluss in Richtung der KMV-Synthese zu leiten, wurden die Gene *hom*^{FBR}, *thrB*, *thrC* und *ilvA*, welche für eine deregulierte Version der Homoserin Dehydrogenase, die Homoserinkinase, die Threoninsynthase und die Threonindehydratase kodieren, auf Plasmiden überexprimiert. Der auf diese Weise entstandene Stamm *C. glutamicum* DM1729 Δ*ilvE* (pEK *hom*^{FBR} *thrB thrC*) (pMM36ptac *ilvA*) ist der erste beschriebene Stamm der speziell zur KMV-Produktion aus Glucose konstruiert wurde. Die ausgeschiedenen KMV-Mengen waren zunächst niedrig, doch durch Verbesserungen der Medienzusammensetzung und der Wachstumsbedingungen konnten höhere Konzentrationen erreicht werden. Unter Limitierung der verzweigtkettigen Aminosäuren wurde eine maximale KMV-Konzentration von 35 mM KMV aus 4 % Glucose hergestellt.

Side Project and Publication

In a side project the regulation of the glycogen synthesis in *C. glutamicum* in dependence of the carbon source was investigated. If grown on glucose *C. glutamicum* accumulates high amounts of glycogen, while glycogen synthesis is very low on the gluconeogenetic substrate acetate. This regulation is at least partially due to the transcriptional regulation of *glgC*, encoding ATP-glucose pyrophosphorylase, the first enzyme of the glycogen synthesis. The transcriptional regulators RamA and RamB were revealed to be part of the regulation mechanism. The collaboration with this project resulted in a co-authorship with the following publication:

Seibold, G. M., C. T. Hagmann, M. Schietzel, D. Emer, M. Auchter, J. Schreiner, and B. J. Eikmanns. 2010. The transcriptional regulators RamA and RamB are involved in the regulation of glycogen synthesis in *Corynebacterium glutamicum*. *Microbiology* **156:** 1256-1263.

Danksagung

Mein besonderer Dank gilt Herrn Professor Bernhard J. Eikmanns, der es mir ermöglicht hat unter seiner Betreuung diese Doktorarbeit anzufertigen. Vor allem möchte ich mich für die angenehme Arbeitsatmosphäre bedanken und dafür, dass du fast immer Zeit für Fragen hattest.

Herrn Professor Peter Dürre danke ich für die freundliche Übernahme des Korreferats und die Versuche, mir eine Ernährung ohne DNA-schädigendes Koffein nahezulegen.

Der Evonik Industries AG danke ich für die finanzielle Unterstützung meiner Arbeit.

Für so manchen guten Tipp möchte ich mich bei Bastian Blombach, Marc Auchter und Dominik Meisohle bedanken (bei letzterem auch für so manches „unnütze Wissen“).

Ich danke Katrin Breitinger und Verena Bückle für stets gute Stimmung im Labor 352, sowie auch allen die zusammen mit mir an der KMV-Produktion gearbeitet haben: Tanja Riester, Sonja Messerschmidt, Dennis Hell und Andreas Schwendtner. Auch Andrea Tautzenberger, Melanie Schietzel und Simon Frewert danke ich für die gute Zusammenarbeit. Nicht zu vergessen meine HPLC-Kumpel Stefan „Elb“ Wieschalka und Philipp von Zaluskowski. Besonders möchte ich mich bei Tanja Laslo bedanken, die mir im Laufe unzähliger Tassen Kaffee eine gute Freundin geworden ist.

Bei Petra Dangel, Brigitte Ehrler, Brigitte Zickner und Anne Frank möchte ich mich für die vielen kleinen Dinge bedanken, die einem Doktoranden das Leben leichter machen.

Allen hier nicht namentlich erwähnten Mitarbeitern des Instituts für Mikrobiologie und Biotechnologie danke ich für ihre Hilfsbereitsschaft und für die vielen geselligen Stunden bei gemeinsamen Aktivitäten und in der Kaffeeecke der Abteilung.

Zuletzt möchte ich noch meinen Eltern danken für die tolle Unterstützung und den Rückhalt auf dem Weg zur Promotion.

Erklärung

Ich versichere hiermit, dass ich die Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, sowie die wörtlich oder inhaltlich übernommenen Stellen als solche kenntlich gemacht habe.

Weiterhin erkläre ich, dass die vorliegende Arbeit weder vollständig noch in Auszügen einer anderen Fakultät mit dem Ziel vorgelegt worden ist, einen akademischen Titel zu erwerben. Ich bewerbe mich hiermit erstmalig um den Doktorgrad der Naturwissenschaften der Universität Ulm.

Ulm, den

……

Vorname Nachname / Unterschrift