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REGULATION OF L-VALINE BIOSYNTHESIS IN *CORYNEBACTERIUM GLUTAMICUM* – ASPECTS OF PHYSIOLOGY AND BIOCHEMISTRY

Doctoral thesis

Riga 2010

ANOTĀCIJA

Darbā pētīta optimālā fizioloģija un iekššūnas bioķīmija, kas nodrošina L-valīna virssintēzi *Corynebacterium glutamicum* ATCC 13032 rekombinanto celmu šūnās. Pētījumi veikti periodiskos un periodiskos ar piebarošanu kultivēšanas apstākļos bioreaktorā, kā arī īstermiņa eksperimentos kolbās.

Pētījumā parādīts, ka pārejas stadija no šūnu maksimālā augšanas ātruma uz zemāku ir optimāls šūnu fizioloģiskais stāvoklis, lai palielinātu L-valīna sintēzi: šūnu augšanas ātruma samazināšanās korelē ar L-valīna sintēzes ātruma (q_P) palielināšanos.

Tālāk tika pētīts q_P palielināšanās bioķīmiskais pamatojums. Tā kā q_P palielināšanās korelēja ar piruvāta iekššūnas koncentrācijas un acetohidroksiskābes sintāzes (AHAS) aktivitātes palielināšanos, bija nepieciešams noskaidrot L-valīna priekšteču un sintēzes ķēdes fermentu lomu L-valīna sintēzes regulācijā. Noskaidrots, ka q_P palielināšanās iespējamais cēlonis ir AHAS aktivitātes palielināšanās, bet piruvāta koncentrācija nav pirmais limitējošais faktors.

AHAS aktivitātes palielināšanās *C. glutamicum* šūnās korelēja ar L-izoleicīna koncentrācijas samazināšanos. Tas norādīja uz iespējamo stingrās kontroles mehānisma pozitīvo ietekmi uz šī fermenta aktivitātes palielināšanu.

Lai noskaidrotu AHAS palielināšanās cēloni (L-izoleicīna limits vai stingrā kontrole) izmantoti *C. glutamicum rel*$^+$ un *rel*$^-$ celmi. Noskaidrots, ka AHAS aktivitātes palielināšanos, kā arī šūnu augšanas ātruma samazināšanos izraisīja L-izoleicīna limits, nevis stingrās kontroles inducēšana. Tomēr šīs kontroles inducēšana nodrošināja palielināta L-valīna sintēzes ātruma uzturēšanu stacionārās fāzes laikā.

Balstoties uz atklātajiem L-valīna virssintēzes palielināšanās principiem, izstrādātas metodes, kuru pielietošana nodrošināja palielinātu q_P *C. glutamicum* šūnās periodiskos ar piebarošanu kultivēšanas apstākļos. Uzturot šūnu augšanas ātrumu virs 0.05 h^{-1} stacionārajā augšanas fāzē ar L-izoleicīna pulsa metodi un optimizējot aerācijas intensitāti, L-valīna sintēzes ātrums palielinājās 2-3 reizes un L-valīna koncentrācija 24 h laikā sasniedza 16 g l^{-1}.

Promocijas darbs izstrādāts 2003. - 2008. gadā LU Mikrobioloģijas un Biotehnoloģijas institūtā. Darbs uzrakstīts uz 103 lapām, satur 18 attēlus un 7 tabulas, apskatīti 174 literatūras avoti.

ANNOTATION

The optimum cellular physiology and intracellular biochemistry to achieve L-valine overproduction by *Corynebacterium glutamicum* ATCC 13032 recombinant strains were investigated in this study. The investigation was carried out under batch and fed-batch cultivation conditions in the bioreactor as well as during short-term experiments in shake flasks.

A transition in the bacterial growth rate to below maximum was found to be an optimum parameter of cellular physiology to increase L-valine synthesis: the decrease in the cellular growth rate correlated with the increase in L-valine synthesis rate (q_P).

The biochemical basis for the increase in q_P was further investigated. As the increase in q_P correlated with the increase in the pyruvate intracellular concentration and acetohydroxyacid synthase (AHAS) activity, the role of L-valine precursors and forming enzymes in L-valine synthesis regulation had to be elucidated. It was found that the increase in AHAS activity was the possible reason for the increase in q_P, but the pyruvate concentration is not the first limiting factor.

The increase in AHAS activity in *C. glutamicum* cells correlated with the decrease in L-isoleucine concentration. It was an indication that the stringent response could be involved in this enzyme upregulation.

To elucidate the condition responsible for the increase in AHAS activity (L-isoleucine limitation or the stringent response induction) *rel*$^+$ un *rel*$^-$ *C. glutamicum* strains were used. It was shown that the L-isoleucine limitation but not the stringent response induction was a condition responsible for the increase in AHAS activity (as well the decrease in the cellular growth). However, the stringent response induction provided an increased L-valine synthesis rate during stationary growth phase.

Basing on the estimated principles of the increase in L-valine overproduction, technologies to maintain an increased L-valine synthesis rate in *C. glutamicum* cells were developed. If the cellular growth rate was maintained above 0.05 h^{-1} during late stationary growth phase with a L-isoleucine pulse and after the optimisation of aeration intensity, q_P increased 2-3 -fold and the final L-valine concentration 16 g l^{-1} was achieved.

The research was carried out in 2002-2008 at the Institute of Microbiology and Biotechnology, University of Latvia. The thesis contains 103 pages. It is supplemented by 18 figures and 7 tables, 174 literature references are discussed.

ABBREVIATIONS

AC – aconitase

AHAS - acetohydroxyacid synthase

AHAIR - acetohydroxyacid isomeroreductase

BCAAs - branched-chain amino acids

DHAD - dihydroxyacid dehydratase

G-6-PDH – glucose-6-phosphate dehydrogenase

LDH – lactate dehydrogenase

NADPH – nicotine adenine dinucleotide phosphate reduced

ppGpp - guanosine 5'-diphosphate 3'-diphosphate

PPP – pentose phosphate pathway

TCA – tricarboxylic acid cycle

TmB – L-glutamate dependent transaminase B

X – biomass concentration (g l^{-1})

μ - specific cellular growth rate (h^{-1})

q_P - specific amino acid synthesis rate (g amino acid g cdm^{-1} h^{-1})

q_S – specific substrate consumption rate (g glucose g cdm^{-1} h^{-1})

$Y_{P/S}$ – L-valine experimental yield (g L-valine g $glucose^{-1}$)

$Y_{X/S}$ – biomass experimental yield (g cdm g $glucose^{-1}$)

Q_P – L-valine productivity (g l^{-1} h^{-1})

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INTRODUCTION

The amino acid production era with *Corynebacterium glutamicum* started in 1956 when the glutamic acid excreting *C. glutamicum* strain was isolated (Kinoshita et al. 1957). Further it was found that mutants of this species could overproduce also L-lysine, L-arginine, L-ornithine, L-threonine and other amino acids. Nowadays bacteria of the genus *Corynebacterium* are among the most important in the biotechnological processes in terms of tonnage and economical value. About 1.5 million tons of L-glutamic acid and 850 000 tons of L-lysine are produced per year with an annual market growth rate of about 7%. However, the production of branched chain amino acids (L-valine, L-isoleucine and L-leucine) does not reach even 1 000 tons per year, and this production rate cannot fulfill the growing market demand (Hermann 2003, Leuchtenberger et al. 2005). Therefore there still is a necessity for the development of highly productive branched chain amino acid microbiological production.

C. glutamicum has some obvious advantages for amino acid synthesis as compared to other bacteria (e.g. to *Escherichia coli* or *Bacillus subtilis*): (i) this bacterium is apathogenic and is classified as GRAS (generally regarded as safe) organism, (ii) this bacterium has small genome size therefore isoenzymes are less abundant and classical genetic engineering methods can be easily applied, (iii) degradative enzymes for many amino acids are absent, (iv) amino acids are exported with transport proteins (Eggeling et al 1997), (vi bacterium tolerates high intracellular concentrations of amino acids.

L-valine is a natural proteinogenic amino acid, and it is one of the essential amino acids for vertebrates. It is mainly used for pharmaceutical purposes and in special dietary nutrition. L-valine is also used as a precursor for the chemical synthesis of herbicides. About 500 tons of L-valine are currently being produced by extraction from acidic hydrolysates of proteins, fermentation and using enzyme membrane reactor technology (Leuchtenberger et al. 2005). The reason why the effective L-valine production has not been developed so far could lie in the complicated control of branched chain amino acid synthesis in bacterial cells: parallel reactions are catalysed by common enzymes and the activity of these enzymes or expression of their encoding genes is feedback controlled by each of branched chain amino acids (Eggeling et al. 1987, Morbach et al. 2000).

Although the big progress has been made in L-valine overproducing strain construction, an understanding in the optimum cellular physiology and biochemistry and methods how to maintain this optimum state is required to control the processes of L-valine microbial synthesis and to achieve L-valine production in high concentrations at high yield from carbon substrates.

Hence, it is very important to deepen the knowledge about the L-valine synthesis control and to find methods to increase L-valine overproduction in bacterial cells.

The aim of the recent work was to characterise optimum physiology and intracellular biochemistry to achieve L-valine overproduction by *C. glutamicum* recombinant strains.

Therefore several tasks for the work were defined:

- to evaluate capability of L-valine synthesis by different *C. glutamicum* recombinant strains,
- to characterise relationship between the cellular physiological state and L-valine overproduction,
- to characterise biochemical basis of the increase in L-valine overproduction (effect of the increase in intracellular precursor availability and effect of the increase in the activity of L-valine forming enzymes),
- to characterize the effect of stringent response on the increase in L-valine overproduction,
- to optimise cultivation conditions in order to maintain optimal physiological state and to maximize L-valine synthesis rate and productivity during cultivations.

The present work was carried out at the Institute of Microbiology and Biotechnology, University of Latvia, in the laboratory of Biochemistry of Producers lead by Dr. habil. biol. Maija Rukliša.

This research was supported by grants from Latvian Council of Science (01.0165 and 04.1112), FP5 project funded by the European commission ("Valpan", QLRT-2000-00497) and by a grant from the European Social Fund program No. ESS2004/3.

1. REVIEW OF THE LITERATURE

1.1. Genus *Corynebacterium*

Currently genus *Corynebacterium* includes more than 70 species. Both nonmedical (found in soil, cheese smear, dairy products, vegetables, fruits and other sources) and medically relevant groups (isolated from human clinical samples or from animals) are widely disseminated in nature (Liebl 2005).

The hierarchy leading to the genus *Corynebacterium* is class *Actinobacteria*, subclass *Actinobacteridae*, order *Actinomycetales*, suborder *Corynebacterineae*, family *Corynebacteriaceae* (Stackebrandt et al. 1997).

The characteristic features of the genus *Corynebacterium* are the following: gram-positive, asporogenous, nonmotile, straight or slightly curved rods, ovals or clubs, often with metahromic granules, often exibit typical V-shaped arrangement of cells, aerobic to facultatively anaerobic, catalase-positive, chemoorganotrophic, predominant cell wall sugars are arabinose and galactose, most species have between 51 and 68 mol% G+C (DNA base composition) (Prescott 1996).

Corynebacterium belongs to a distinct and well-separated group within the Gram-positive bacteria characterised by a unique cell wall organization and possession of unique cell wall components not present in other Gram-positives. In addition to the inner membrane, the cell envelope is constituted of a thick arabinogalactan-peptidoglycan polymer covalently linked to an outer lipid layer, which is mainly composed of mycolic acids and probably organized in an outer membrane like structure (for review see Dafffe 2005). This group also includes genera *Dietzia*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* (Pascual et al. 1995). Thereby non-pathogenic *Corynebacterium* strains could serve as model organisms for such well-known pathogens as *Mycobacterium tuberculosis, Mycobacterium leprae* and *Corynebacterium diphtheriae*.

Along with *C. glutamicum*, other industrially relevant species are *C. callunae*, *C. efficiens*, *C. thermoaminogenes* and *C. ammoniagenes* (Liebl 2005).

1.2. *Corynebacterium glutamicum* and its application in biotechnology

C. glutamicum (former *Micrococcus glutamicus*) was first isolated from soil by Kinoshita et al. in 1956 in a screen for bacteria that excrete L-glutamate (Kinoshita et

al. 1957). To the species belong also formerly wrong classified type strains as *Brevibacterium flavum*, *B. glutamigenes*, *B. lactofermentum* and others (Liebl 2005).

Natural habitats of the species are soil, sewage and manure, vegetables and fruits. The *C. glutamicum* type strain ATCC 13032, which nowadays is widely used for amino acid overproducing strain construction, was originally isolated from sewage. *C. glutamicum* can grow well at 30°C in different complex media – from standard peptone-yeast extract media to very rich media such as Brain Heart Infusion. *C. glutamicum* can grow also in chemically defined minimal media, only the species is auxotrophic for D-biotin (Liebl 2005). *C. glutamicum* can use glucose, fructose, sucrose, ribose, maltose and citrate (Dominguez et al. 1997; Kiefer et al. 2002; Wendisch 2003, Polen et al. 2007) as well as acetate, pyruvate, lactate, and glutamate (Kramer et al. 1990; Gerstmeir et al. 2003) and also ethanol (Arndt et al. 2008) as a carbon source.

As it became important for the industry, *C. glutamicum* has been investigated intensively in recent decades. Central carbon metabolism, anaplerotic reactions, and several amino acid biosynthesis pathways, as well as many transport processes, have been studied in great detail (for review see Eggeling and Bott 2005).

The complete genome sequence of *C. glutamicum* ATCC 13032 was determined in independent approaches by at least three research teams. Two complete genomic sequences for the strain *C. glutamicum* are available today (Kalinowski et al. 2003, Ikeda and Nakagawa 2003). The *C. glutamicum* genome is represented by a single circular chromosome of 3,28 Mbp in size. Average G+C content is 53.8 %, which is close to that of *E. coli* and at lower boundary for the taxonomic class of the *Actinobacteria* (Kalinowski et al. 2003, Kalinowski 2005).

Due to the available extensive knowledge, there have been developed a lot of techniques and tools for genetic engineering (Kirchner and Tauch 2003) and genome-based technologies, such as DNA-microarray technology (Wendisch 2003) and proteomics (Schaffer et al. 2001, Schaffer and Burkovski 2005).

L-lysine and L-glutamate producing strains are being constantly improved by metabolic engineering and DNA-microarray technologies (Uy et al. 2005, Ohnishi and Ikeda 2006; Becker et al 2007; Sindelar and Wendisch 2007, Blombach et al. 2009, Blombach and Seibold 2010). Because of the market development there is also focus on the development of other amino acid production by *C. glutamicum* such as

L-glutamine (Kusumoto 2001), L-tryptophan (Ikeda 2006), L-cysteine (Wada and Takagi 2006), L-isoleucine (Morbach et al. 1996, Eggeling et al. 1997), L-serine (Stolz et al. 2007), L-alanine (Jojima et al. 2010) and L-valine (Sahm and Eggeling 1999, Radmacher et al. 2002, Elišáková et al. 2005, Blombach et al. 2007[b], 2008, Bartek et al. 2010[a], Bartek et al. 2010[b], Holatko et al. 2009).

The use of *C. glutamicum* does not stop only with amino acid production. *C. glutamicum* could be used also for organic acid production such as lactic and succinic acid (Okino et al. 2005) and for xylitol microbiological production (Sasaki et al. 2010). Another target could be ethanol production (Inui et al. 2004), even using such substrates as xylose (Sakai et al. 2007). Some investigations point on *C. glutamicum* potential use in biosorption and bioremediation (Choi and Yun 2004, Girbal et al. 2000, Mateos et al. 2006).

1.3. Characterization of central metabolism and electron transport in *C. glutamicum* and their role in amino acid synthesis

When talking about amino acid overproduction it is not enough to pay attention only to a distinct amino acid synthesis pathway. Many other cell processes are linked to the amino acid synthesis. To achieve the amino acid overproduction the cell has to (i) provide the basic and intermediate building blocks and redox equivalents (e.g. NADPH) via central metabolism, (ii) provide energy (ATP) via respiratory chain, (iii) tolerate high intracellular concentrations of synthesized amino acid, (iv) the desired end product has to be secreted into the culture medium (Fig 1).

Besides, the cell has to have facility to switch metabolic fluxes towards the preferable amino acid synthesis. Consequently, knowledge about the central metabolism, energy generation, product synthesis and export is essential to understand the amino acid overproduction.

Glycolysis, the pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA), and glyoxylate cycle have been established as the central metabolism pathways in *C. glutamicum* while the Entner-Doudoroff pathway is not functioning (Fig. 2) (Yokota and Lindley 2005).

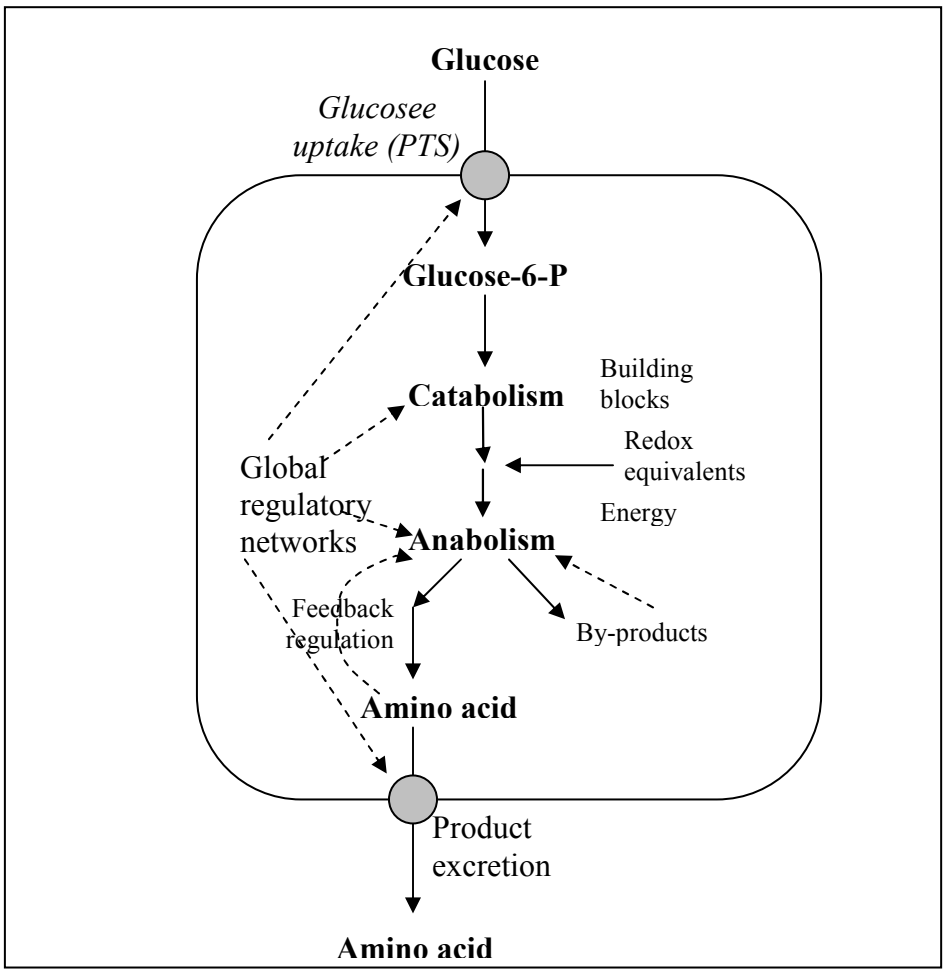

Fig. 1. Schematic drawing of important steps in amino acid production by a bacterial cell. After Krämer (1996). Dashed line indicates regulatory network.

C. glutamicum possesses at least three phosphotransferase systems (PTSs) enabling the uptake of glucose, fructose and sucrose (Yokota and Lindley 2005). Unlike *E. coli* and *B. subtilis* that show pronounced catabolite repression (ie. utilize one carbon source preferentially, with the further carbon source(s) being consumed only, when the preferred one is exhausted), *C. glutamicum* typically does not show diauxic growth or preferential carbon source utilization on mixtures of carbon sources, but co-utilizes the carbon sources present in the mixtures (Wendisch et al 2000, Paegle and Ruklisha 2003, Wendisch 2006). Therefore the various PTSs of *C. glutamicum* are expressed constitutively and enable simultaneous co-consumption of sugars from mixtures (Dominguez et al. 1997). Besides the PTSs not only enable carbon source transportation into the cell but also produce pyruvate in group translocation reaction.

Unlike many microorganisms, under normal growth on glucose, the carbon flux distribution between glycolysis and the PPP has been shown to be slightly in favour of

the PPP in *C. glutamicum.* The most important control point in glycolysis is pyruvate kinase. It is inhibited by ATP and activated by AMP (Ozaki and Shiio 1969, Jetten et al. 1994). Thus glycolysis is controlled not directly by the intermediary metabolites but rather by the energy status of the cell. The main role of glycolysis in amino acid synthesis is the production of pyruvate and 3-phosphoglicerate, significant precursors for amino acid synthesis (pyruvate and serine family, respectively).

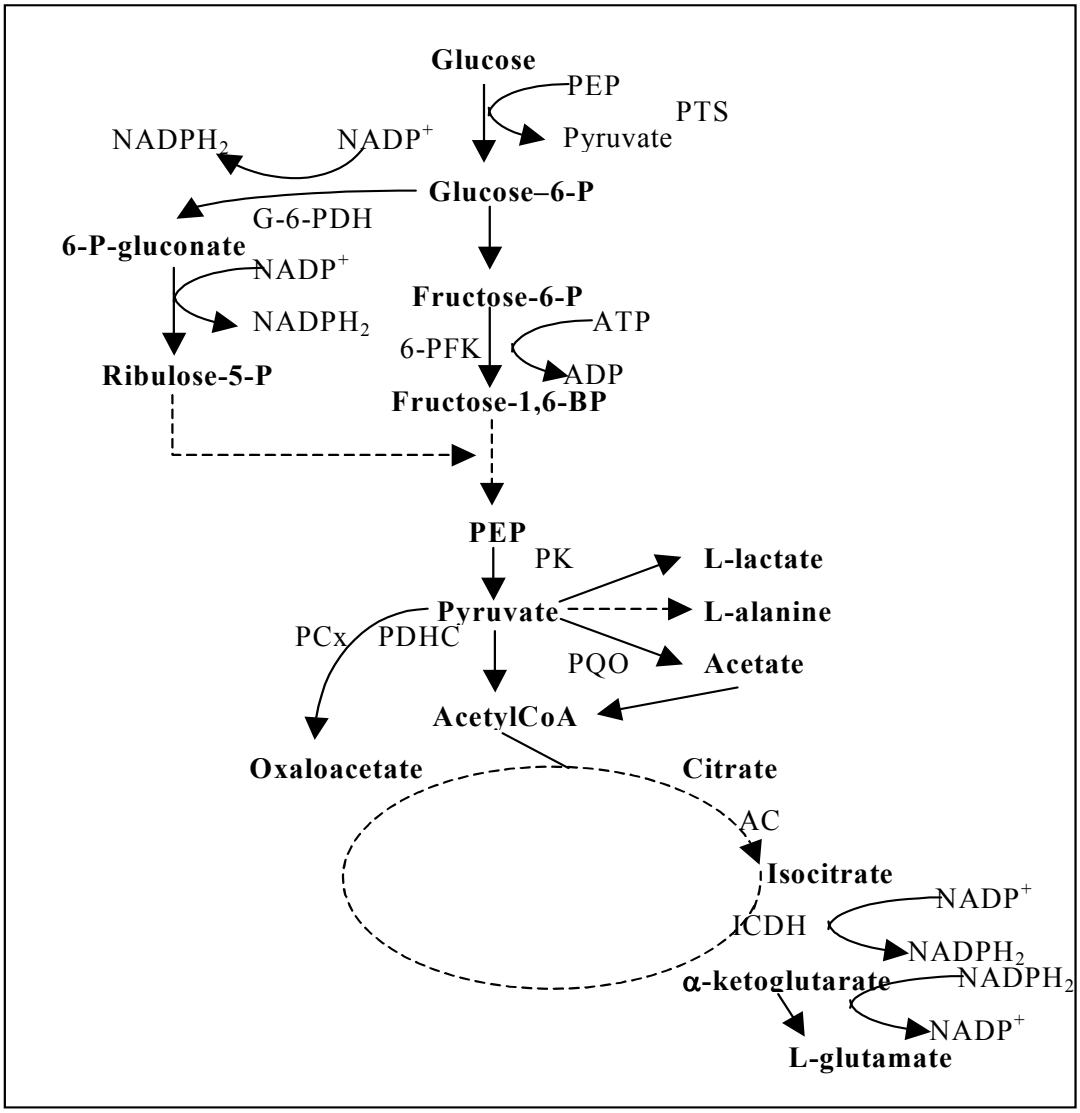

Fig. 2. Simplified scheme of the central metabolism in *C. glutamicum.*

Enzymes of central metabolism**:** PTS, phosphoenolpyruvate:glucose phosphotransferase; PGI, phosphoglucose isomerase**;** PK, pyruvate kinase; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex, PQO, pyruvate:quinone oxidoreductase; G-6-PDH, glucose–6–phosphate dehydrogenase; 6-PFK, 6-phosphofructokinase; AC, aconitase; ICDH, isocitrate dehydrogenase; GDH, $NADP^+$ specific L-glutamate dehydrogenase.

Key enzyme of the PPP is glucose-6-phosphate dehydrogenase (G-6-PDH). G-6-PDH is feedback inhibited by NADPH (Moritz et al. 2000). The PPP is mainly regulated by the ratio of NADPH and $NADP^+$ concentration (not by the concentration of NADPH itself) via the specific enzyme activity of G-6-PDH (Moritz et al. 2000). The main role of the PPP in amino acid synthesis is to provide the cofactor NADPH and precursors ribose-5-phosphate and erythrose-5-phosphate.

Independent of the carbon and energy sources used, the tricarboxylic acid (TCA) cycle or at least part of it must be active in *C. glutamicum.* TCA cycle in *C. glutamicum* is subject to a complex regulation at the transcriptional and post-transcriptional levels (Bott 2007, Emer et al. 2009). Although citrate synthase (CS) has been considered as TCA regulatory enzyme in many bacteria, it was shown that in *C. glutamicum* neither the synthesis nor the activity of CS is subject to severe regulation (Eikmanns et al. 1994). Uncommonly aconitase (AC) was recognized as a major control point of tricarboxylic acid cycle (TCA) activity in *C. glutamicum* (Ruklisha et al. 1987, Krug et a. 2005).

C. glutamicum is also characterized by a surprisingly diverse set of reactions at the anaplerotic node of TCA. It possesses both a phosphoenolpyruvate (PEP) carboxylase (Eikmanns et al. 1989) and a pyruvate carboxylase (Peters-Wendisch et al. 1997), which synthesize oxaloacetate. TCA provides energy for cell and biosynthetic precursors for the aspartate and glutamate family of amino acids. Although NADPH is produced in TCA as well, the carbon flux analysis in a L-lysine-producing *C. glutamicum* strain revealed that in minimal medium with glucose as carbon source TCA plays a minor role for the generation of NADPH (Marx et al. 1996, 1997, 1999, 2003).

C. glutamicum possesses a branched electron transport chain to oxygen (Fig. 3). Reducing equivalents obtained by the oxidation of various substrates are transferred to menaquinone. From menaquinone the electrons are passed either via the cytochrome bc_1 complex to the aa_3-type cytochrome *c* oxidase with low oxygen affinity, or to the cytochrome *bd*-type menaquinol oxidase with high oxygen affinity. The bc_1-aa_3 branch, but not the *bd* branch, is of a major importance for aerobic growth in minimal medium (Bott and Niebisch 2003). The complete aerobic oxidation of glucose via glycolysis, TCA and the bc_1-aa_3 branch of the respiratory chain would result in the formation of 4 ATP by substrate level phosphorylation and of 22.7 ATP by oxidative

phosphorylation. The use of the cytochrome *bd* branch would result in the synthesis of only 6.7 ATP by oxidative phosphorylation (Bott and Niebisch 2003).

The main electron transport chain (ETC) can be blocked by KCN (or other inhibitors) therefore the term "cyanide-sensitive respiration" is sometimes used. If the main ETC is not active, alternative electron transport pathways, which characterize oxygen consumption uncoupled to oxidative phosphorylation ("cyanide resistant respiration"), become active (Toma et al. 1984). Changes of the efficiency of oxidative phosphorylation caused by qualitative changes of the respiratory chain were found to have strong effects on the growth and metabolism in general and the substrate consumption rate and amino acid production in particular (Ruklisha et al. 1981, Toma et al. 1984, Bott and Niebisch 2003).

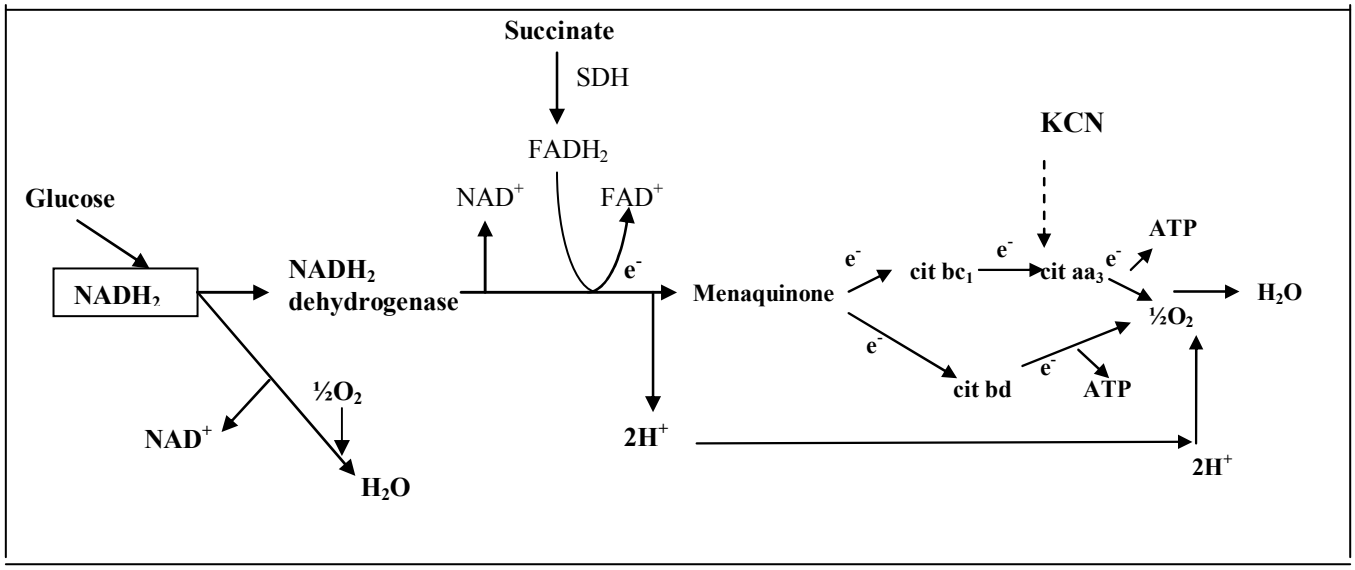

Fig. 3. Schematic overview on the respiratory chain of *C. glutamicum.* The dashed line indicates inhibitor action point. After Ruklisha et al. 1993 and Bott and Niebisch 2003.

The flux studies in *C. glutamicum* showed an enormous flexibility within the central metabolism – ability to synthesize precursors via different pathways, rearrangement of metabolic fluxes in desired direction, respond to environmental changes by rearrangement of energy generation and increase in substrate consumption rate. All these properties make *C. glutamicum* one of the best amino acid producing bacteria.

1.4. Regulation of amino acid overproduction in *C. glutamicum*

Methods to achieve certain amino acid overproduction usually are based on (i) the increase in amino acid precursor supply, and (ii) the increase in amino acid synthesis pathway activity. Amino acid precursor availability could be increased by (i) altering cell physiology, (ii) blocking pathways leading to by-product synthesis, (iii) amplification of precursor synthesis enzymes. Amino acid synthesis pathway activity could be increased by (i) the amplification of amino acid synthesis enzymes, (ii) the introduction of feedback resistant enzymes.

It is necessary to find the bottleneck in the amino acid synthesis pathway because not all the steps in synthesis pathway might be limiting. For example, all six enzymes from L-lysine synthesis pathway were analysed in order to evaluate their role in L-lysine synthesis. The analysis of L-lysine formation revealed that an overexpression of the feedback-resistant aspartate kinase alone suffices to achieve L-lysine formation while amplification of other L-lysine enzymes had no effect on amino acid overproduction (Cremer et al. 1991). Besides often it is not enough to increase only amino acid synthesis enzyme availability, it is necessary to provide sufficient concentration of amino acid precursors. A high increase in amino acid synthesis could be achieved by a simultaneous increase in the intracellular precursor and amino acid synthesis enzyme availability. The single overexpression of either pyruvate carboxylase or aspartate kinase, e.g. enzyme of central metabolism and that of L-lysine production pathway, did not result in the L-lysine synthesis increase. The simultaneous overexpression of both enzymes resulted in more than 250% increase of the L-lysine specific productivity (Koffas et al. 2003).

1.4.1. Regulation by precursor availability

It is necessary to pay attention to certain amino acid precursor supply because in order to achieve high amino acid yields the task is to direct as much carbon as possible from sugar to the desired amino acid.

There could be several approaches to increase precursor availability for amino acid synthesis during cultivations. The first approach could be the application of specific cultivation strategies in order to control cell physiology - to restrain growth rate and respectively reduce biomass formation, and consequently obtain increased precursor availability for amino acid synthesis.

One of the methods to regulate cellular growth rate is to limit supply of some medium components, thereby restricting the growth and extending the period of increased product accumulation. It was shown that improved L-lysine fermentation performance by *C. glutamicum* could be achieved through fed-batch cultivation of the organism under conditions of L-threonine limitation. The maximum L-lysine production was achieved while allowing biomass formation just sufficient to maintain the L-lysine synthesis (Kiss and Stephanopoulos 1991, Ruklisha et al 1992). The growth inhibition of D-pantothenate auxotroph *C. glutamicum* subsp. *lactofermentum* by limiting the supply of D-pantothenate resulted in increased rates of L-lysine synthesis. It was explained by the restricted growth and redirected flow of carbon to L-lysine rather than to L-isoleucine or L-valine (An et al. 1999). In L-leucine auxotroph L-lysine overproducing *C. glutamicum* strains cell growth and L-lysine synthesis was controlled by L-leucine concentration (Patek et al. 1994, Takiguchi et al. 1998).

The increased availability of the necessary precursor can be achieved also indirectly with intracellular limitation. The overexpression of certain amino acid biosynthesis gene sometimes does not result only in the increased availability of this gene product but also can redirect metabolic fluxes. Eggeling et al. (1998) reported that increased lysine production due to the overexpression of *dapA* gene (encoding dihydrodipicolinate synthase from L-lysine synthesis pathway) decreased the carbon flux to the branch of L-threonine and increased the intracellular levels of pyruvate in *C. glutamicum.* The decreased flux towards threonine caused threonine intracellular limitation and consequently a growth rate reduction that resulted in increased availabilities of metabolites within the central metabolism (Eggeling et al. 1998).

We should also consider cases when the same precursor may be synthesized in various metabolic pathways. It was shown by Shvinka et al. (1980) that L-lysine experimental yield ($Y_{P/S}$) could vary a lot depending on the pathway generating precursors. If the oxaloacetate was synthesized in TCA, the L-lysine experimental yield was 0.40, but if the oxaloacetate was synthesized by PEP-carboxylase, the $Y_{P/S}$ was 0.69 g L-lysine g glucose, respectively.

A significant increase in precursor availability can be achieved by the amplification of precursor synthesis enzymes. For example, the increase of oxaloacetate (precursor of L-lysine) supply was achieved by the overexpression of the pyruvate carboxylase gene in L-lysine overproducing *C. glutamicum* (Peters-

Wendisch et al. 2001). Increased pyruvate carboxylase activity resulted in approximately 50% higher L-lysine accumulation.

Another method to increase the intracellular precursor availability is to avert precursor use in competing metabolic pathways by certain genetic alterations. The importance of pyruvate availability was demonstrated by inactivation of the pyruvate dehydrogenase complex in L-lysine producing *C. glutamicum* strain (Blombach et al. 2007[a]). Mutant with the deleted *aceE* gene encoding the E1p enzyme of the pyruvate dehydrogenase complex showed more than 40% higher L-lysine yield. Increased oxaloacetate supply for L-lysine synthesis was achieved by inactivation of PEP carboxykinase and thus eliminated carbon flow in gluconeogenic pathway (Riedel et al. 2001).

Cofactor NADPH is also important in the amino acid synthesis because it provides reducing equivalents for biosynthetic reactions. If only 0.018 M NADPH is needed for the synthesis of 1 g cell dry weight (Sonntag et al. 1995) then 4 moles of NADPH is required to synthesize 1 mole of L-lysine, and 2 moles of NADPH is required to synthesize 1 mole of L-valine.

NeverthelessNADPH is not always considered as a limiting condition in amino acid synthesis. It was elucidated that NADPH availability was not rate limiting in L-lysine synthesis in several investigations using various *C. glutamicum* L-lysine overproducers (Marx et al 1999, Moritz et al. 2000, 2002). However other data indicate that NADPH availability is an essential parameter to achieve L-lysine overproduction (Ruklisha et al. 1992). Recently G6P dehydrogenase encoding gene *zwf* was overexpressed in L-lysine overproducing *C. glutamicum* strain in order to increase NADPH availability. The metabolic flux analysis revealed a 15% increase of PPP flux in response to the overexpression of the *zwf* gene. Increased PPP activity resulted in the increased L-lysine synthesis (Becker et al. 2007). Also the disruption of the phosphoglucose isomerase and thus redirection of the carbon flux from glycolysis towards the PPP resulted in increased L-lysine formation (Marx et al. 2003). Increased flux from glycolysis to the PPP and, thus, increased NADPH supply was achieved also by the overexpression of fructose 1,6-biposphatase (Becker et al.2005). Consequently although the NADPH concentration will not always be limiting, the role of NADPH availability should not be neglected.

1.4.2. Regulation by amino acid synthesis pathway activity

An increase in the amino acid overproduction can be achieved by altering the specific amino acid biosynthesis pathway (i) by increasing the availability of amino acid synthesis enzymes, (ii) by altering the regulation pattern e.g. loss of feedback control.

It is known that aspartate kinase, which is the first enzyme to convert the metabolite oxaloacetate to L-lysine, L-methionine, L-threonine, and L-isoleucine, is multivalently feedback inhibited by L-lysine plus L-threonine (Shiio and Miyajima 1969). Therefore a significant increase in L-lysine synthesis was achieved by the overexpression of *lys*

C gene encoding the feedback resistant aspartate kinase and *dapA* gene encoding dihydrodipicolinate synthase (Cremer et al. 1991). The overexpression of the *dapF* (encoding diaminopimelate epimerase) and *dapC* (encoding succinyl-aminoketo-pimelate transaminase) genes of the succinylase branch of L-lysine synthesis pathway in an industrial *C. glutamicum* strain resulted in further increase in L-lysine production (Hartmann et al. 2003).

However, a part of complexity associated with amino acid synthesis arises from the fact that different amino acid synthesis pathways are rather closely linked together – they consume the same precursors, or similar enzymes catalyse reactions for various amino acids. For example, L-lysine, L-methionine, L-threonine, and L-isoleucine are derived from aspartate, and cells have to regulate the flux among these competing pathways. These mechanisms can be used to channel the carbon flux towards the preferable amino acid. The flux from L-lysine was shifted towards L-threonine due to the amplification of homoserine dehydrogenase, feedback resistant to L-threonine and homoserine kinase in a high L-lysine overproducing strain (with deregulated aspartate kinase). It enabled channelling of the carbon flow from the intermediate aspartate semialdehyde towards homoserine, resulting in a high accumulation of L-threonine (Eikmanns et al. 1991, Reinscheid et al. 1994). A significant level of L-isoleucine overproduction was achieved by coupling the strategies for overproducing of L-lysine and L-threonine with the strategies for further metabolizing L-threonine toward L-isoleucine. The carbon flux was diverted by the amplification of homoserine dehydrogenase, feedback-resistant to L-threonine and threonine dehydratase, feedback resistant to L-isoleucine in L-lysine overproducing *C. glutamicum* strain (Morbach et al. 1995, Morbach et al. 1996).

Besides, feedback inhibition of amino acid synthesis enzymes could be eliminated not only by genetic alterations but also by cultivation strategies. As aspartate kinase from L-lysine synthesis pathway is multivalently inhibited by L-lysine and L-threonine, special feeding strategies should be performed during the cultivation of L-threonine auxotroph mutants – L-threonine should be supplied in such an amount it is consumed until the L-lysine concentration reaches 5 mM (Ruklisha et al. 1992).

1.4.3. Regulation by export

The amino acid efflux plays a significant role in amino acid overproduction. It appears that amino acid export could seriously limit the effectiveness of some amino acid production despite the achieved high rates of amino acid synthesis.

Amino acid efflux from *C. glutamicum* cells occurs in two ways: by the passive diffusion, and by the specific excretion systems (most cases) (Burkovski and Krämer 2002). Active export in *C. glutamicum* is demonstrated for L-glutamate (Hoischen and Krämer 1989), L-lysine (Broer and Krämer 1991, Vrljic et al. 1996), L-isoleucine (Zittrich and Krämer 1994, Kennerknecht et al. 2002), L-valine and L-leucine (Kennerknecht et al. 2002), L-threonine (Palmieri et al. 1996) and L-methionine (Trötschel et al. 2005). The export systems require energy for their functioning. However, due to the different physiochemical properties of the individual amino acid, each amino acid displays its own special export properties. L-lysine export is based on the electrochemical gradient and L-lysine is presumably exported in symport with two OH^- ions (Broer and Cramer 1991). L-threonine and BCAAs export is driven by the proton motive force (Palmieri et al 1996, Kennerknecht et al. 2002). For L-lysine and L-glutamate the transport properties are also definitively determined by the outer lipid layer of the cell - L-lysine export was increased in a mutant lacking mycolic acids in the cell wall (Eggeling 2005).

The export was identified as the most limiting step in L-isoleucine production (Morbach et al. 1996) and in L-threonine production (Reinscheid et al. 1994) by *C. glutamicum* recombinat strains, therefore attempts were made to increase the export rate. Increased L-threonine export was achieved by *thrE* (encoding L-threonine exporter) overexpression by up to 40 % (Simic et al. 2001). It was shown that also L-lysine export could be increased due to the overexpression of L-lysine exporter (*lysEG)* (Vrljic et al. 1996). Although the increased export models are convincing, up

to day there are no reports available on successful application of this concept in industrial amino acid production (Burkovski and Kramer 2002).

1.5. L-valine synthesis pathway in *C. glutamicum*

L-valine together with L-alanine and L-leucine belong to the pyruvate-derived family of amino acids. Furthermore L-valine in common with L-isoleucine and L-leucine are so called branched chain amino acids (BCAAs). L-valine and L-isoleucine are synthesized in parallel pathways in which homologous reaction steps are catalysed by the same enzymes. L-leucine is branching off the L-valine pathway.

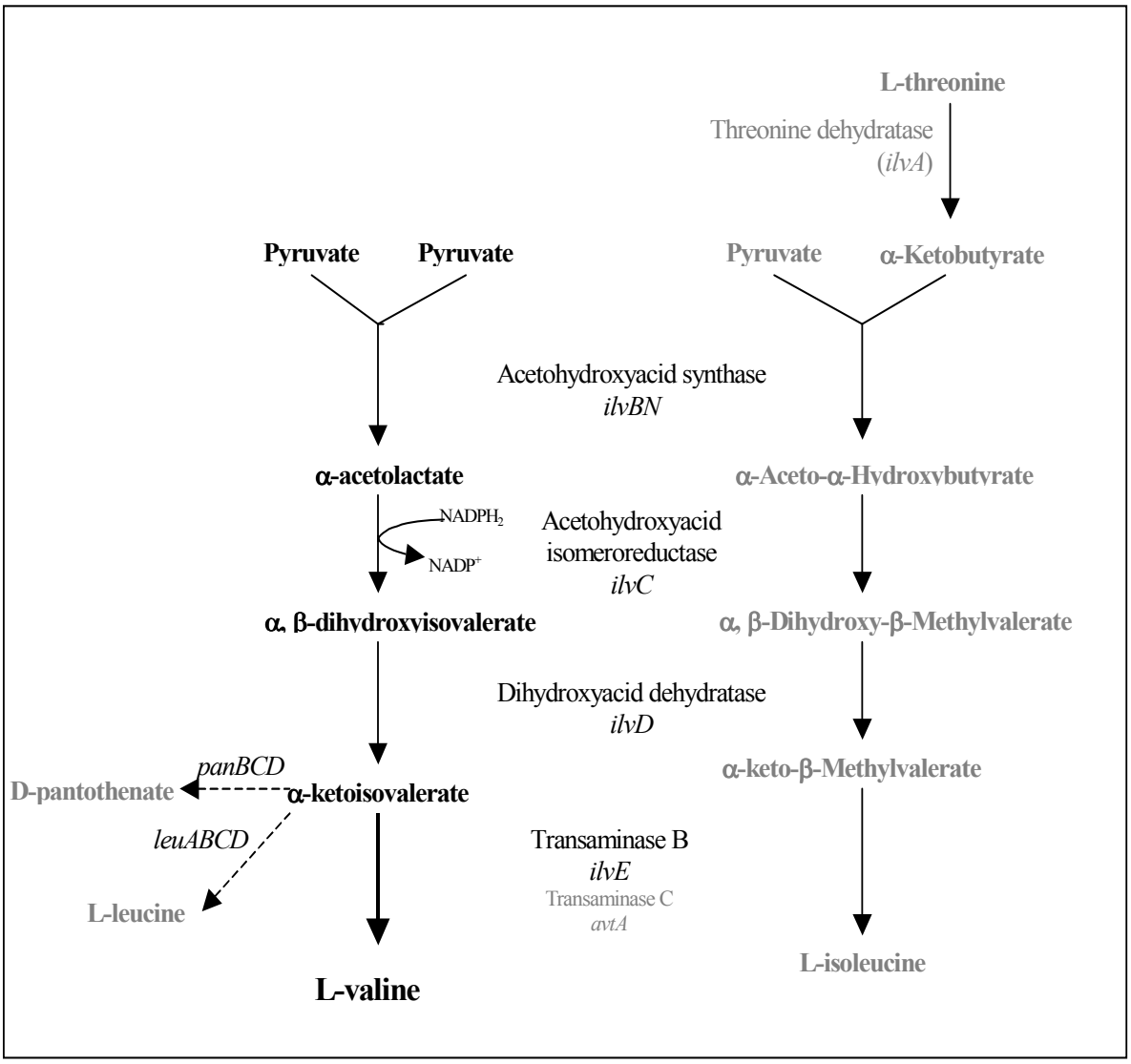

Fig. 4. Simplified scheme of the biosynthetic pathways of L-valine, L-isoleucine, L-leucine and D-pantothenate in *C. glutamicum.* Biosynthesis enzymes encoding genes are given in italics.

Comparing to *Escherichia coli* L-valine synthesis pathway in corynebacteria is less complex due to absence of isoenzymes (Eggeling et al. 1987, Keilhauer et al. 1993). In *E. coli* the biosynthesis of branched-chain amino acids involves a set of reactions including branched and parallel reactions catalysed by common enzymes or isoenzymes, multivalent regulation of both gene expression and allosteric enzymes (Barak et al. 1987).

The metabolic pathway for the L-valine formation consists of four steps (Fig. 4). In first reaction acetohydroxy acid synthase (AHAS, EC 2.2.1.6) catalyzes decarboxylation of pyruvate and its condensation either with another molecule of pyruvate to produce acetolactate (a precursor of L-valine and L-leucine) or with α-ketobutyrate to produce acetohydroxybutyrate (a precursor of L-isoleucine). The enzyme requires three cofactors: thiamine diphosphate, a divalent metal ion such as Mg^{2+} and FAD (Duggleby at al. 2000). Opposite to *E. coli* in *C. glutamicum* only a single AHAS was found (Eggeling et al. 1987; Keilhauer et al. 1993). The active holoenzyme is composed of two large (catalytic) subunits and two small (regulatory) subunits, encoded by the genes *ilvB* and *ilvN*, respectively (Keilhauer et al. 1993).

The acetohydroxy acid isomeroreductase (AHAIR, EC 1.1.1.86) catalyses both isomerisation and reduction steps of the transformation of α-acetolactate to dihydroxyisovalerate. AHAIR is encoded by *ilvC* (Cordes et al. 1992, Keilhauer et al. 1993). The enzyme requires NADPH as cofactor.

In the third step of the L-valine pathway dihydroxy acid dehydratase (DHAD, 4.2.1.9) catalyses the dehydration and the tautomerisation of the D,L-α,β-dihydroxyisovalerate, leading to the formation of α-ketoisovalerate. DHAD is encoded by *ilvD* (Radmacher et al. 2002). α-ketoisovalerate is a precursor also for L-leucine and D-pantothenate.

In the last step, transaminase B (TmB, 2.6.1.42) reversibly transfers one α-amino group from aliphatic amino acids such as L-glutamate to α- ketoisovalerate, leading to the formation of L-valine and α-ketoglutarate. The enzyme requires pyridoxal phosphate as cofactor. TmB is encoded by *ilvE* (Radmacher et al. 2002). Recently, another aminotransaminase, encoded by *avtA*, in *C. glutamicum* was identified. It uses L-alanine as an amino donor, and thus resembles transaminase C in *E. coli* (McHardy et al. 2003; Marienhagen et al. 2005). Thus *C. glutamicum* possess two enzymes with the ability to catalyze the transamination reaction of L-valine biosynthesis (McHardy et al. 2003). But compared to TmB, AvtA has weak affinities

for the branched-chain amino acid intermediates (Leyval et al. 2003, Marienhagen et al. 2005). Thus AvtA could be involved in the adjustment of amino acid pool sizes in *C. glutamicum* rather than fulfilling specific biosynthesis function (Marienhagen et al. 2005).

1.6. Regulation of L-valine biosynthesis in *C. glutamicum*

1.6.1. Regulation of L-valine biosynthesis enzymes

1.6.1.1. Regulation on gene expression level

It was previously shown that AHAS activity was inhibited by all BCAAs (Eggeling et al. 1987). It could be due to (i) regulation of gene transcription or (ii) enzyme feedback inhibition.

AHAS is encoded by *ilvBN* gene, which forms an operon together with *ilvC*. It has been discovered that *ilvBNC* operon transcription is controlled by a translation-coupled attenuation mechanism involving antitermination (Morbach et al. 2000). Typical features for regulation by attenuation for *ilvBNC* operon were shown: formation of a very short abundant RNA corresponding to the prematurely terminated *ilvBNC* transcript and increased formation of *ilvBNC* transcript under growth limitation by all BCAAs or by any of them.

It was shown that in comparison with the expression activity obtained when *C. glutamicum* was grown with an excess (5 mM each) of the BCAAs, activity of the *ilvB* promoter was increased about 2-fold under conditions of growth limitation (0.5 mM each) by any of the branched-chain amino acids. It means that each of the branched chain amino acids alone is involved in *ilvB* expression control. At the same time, expression of the operon was induced in the presence of α-ketobutyrate (the precursor of L-isoleucine) (Keilhauer et al. 1993, Morbach et al. 2000). This was explained with L-valine, L-leucine and D-pantothenate shortage in cell caused by α-ketobutyrate addition. Thus another independent regulation operates in control of *ilvBNC* expression. It was confirmed also by the absence of a ribosome binding site for leader peptide formation. Consequently the complex expression pattern of the *ilvBNC* operon and the respective control mechanisms still need to be revealed (for review see Pátek 2007).

Despite the close linkage *ilvC* is expressed independently of *ilvB* (Cordes et al. 1992). It was shown that contrary to *ilvBN* and *ilvNC* transcripts, the amount of which was increased in the presence of α-ketobutyrate, *ilvC* transcript was present in large

excess under all conditions assayed. It means that additional promoters reside within the *ilvBNC* operon that direct the transcription of either *ilvBNC* together, *ilvNC*, or *ilvC* alone (Keilhauer et al. 1993). Recently it was shown that *ilvC* also exhibits ketopantoate reductase activity. This double function may explain why *ilvC* shows the highest transcription rate out of three genes in the *C. glutamicum ilvBNC* operon (Merkamm et al. 2003).

Little is known about the transcriptional regulation of *ilvD* and *ilvE*. *ilvD* is separated from the *ilvBNC* operon of *C. glutamicum* by just one ORF. *ilvE* is not a part of an operon or clustered with other genes of the pathway (Radmacher et al. 2002).

1.6.1.2. Regulation on the enzyme activity level

It has been shown that AHAS activity in *C. glutamicum* is regulated by (i) substrate concentration, (ii) feedback inhibition.

Since AHAS catalyses the first reaction in BCAAs biosynthesis pathway, the flow of pyruvate or α-ketobutyrate towards L-valine or L-isoleucine could be dependent on the affinity of the AHAS for respective substrates. Estimated values of K_m for pyruvate and α-ketobutyrate were 8.3 mM (Eggeling et al. 1987, Leyval et al. 2003) and 4.8 mM (Eggeling et al. 1987), respectively. It means that AHAS has a much higher affinity to α-ketobutyrate than to pyruvate and therefore in the presence of α-ketobutyrate the synthesis of L-isoleucine is preferred, but the synthesis of L-valine is reduced. Moreover, because pyruvate is a key metabolite of the central metabolism, AHAS still competes with several enzymes to transform pyruvate. For example $K_{m\text{-pyruvate}}$ for pyruvate carboxylase is 1.3 mM (Peters-Wendisch et al. 1997), $K_{m\text{-pyruvate}}$ for pyruvate dehydrogenase is 1.7 mM (Schreiner et al. 2005). Another significant pyruvate consuming enzyme is lactate dehydrogenase. Unfortunately the K_m value for lactate dehydrogenase of *C. glutamicum* is not yet available. However it can be assumed that the pyruvate flux through lactate dehydrogenase could be significant under specific cultivation conditions.

It has been shown that 5mM L-valine, L-isoleucine and L-leucine cause a decrease in AHAS activity in *C. glutamicum* for 50%, 45% and 27% respectively (Eggeling et al. 1987). In further investigation the IC_{50} concentrations for AHAS with L-valine, L-isoleucine, and L-leucine were determined (0.9, 3.1, and 6 mM, respectively) (Leyval et al. 2003). Therefore it is obvious that L-valine has a more

severe effect on AHAS activity. However no cooperative inhibiting effect of the different branched-chain amino acids on the AHAS activity was found – in the presence of different combinations of two or three BCAAs AHAS inhibition did not exceed 50% – 57% (Eggeling et al. 1987, Elišáková et al. 2005). This indicated that a single binding (allosteric) site for the three amino acids is present on the enzyme (Elišáková et al. 2005).

To reduce AHAS inhibition by L-valine and other BCAAs, an AHAS protein resistant to inhibition by all BCAAs was constructed by site-directed mutagenesis of *ilvN,* coding for the regulatory subunit of AHAS (Elišáková et al. 2005). Constructed strain *C. glutamicum* Δ*ilvA* Δ*panBilvN*M13 (pECKA*ilvBNC*) was able to accumulate up to 130 mM L-valine (Elišáková et al. 2005).

Compared to AHAS, other L-valine synthesis enzymes are not so strictly regulated. Detected IC_{50} values for AHAIR with L-valine and L-leucine were 6.6 mM and 9.9 mM respectively. L-isoleucine is not inhibiting AHAIR. DHAD appeared to be very weakly inhibited by both L-valine and L-leucine (IC_{50} were, respectively, 170 and 120 mM). As for AHAIR, L-isoleucine is not DHAD inhibitor (Leyval et al. 2003).

1.6.2. Branched chain amino acid export and its regulation

Efflux of BCAAs from *C. glutamicum* cell occurs in two ways: 1) in part via diffusion, 2) by carrier-mediated export, dependent on the proton motive force. BCAAs exporter is a two-component permease BrnFE, encoded by two genes *brnF* and *brnE.* The genes are organized as an operon (Kennerknecht et al. 2002). Recently it was found that BrnFE exports also methionine (Trötschel et al. 2005). For L-isoleucine efflux diffusion and active transport contribute almost equally.

BrnFE has low affinity towards L-isoleucine - K_m= 21 mM. K_m constants for other BCAAs are not determined. Nevertheless, it was shown that L-valine is exported at a significantly reduced rate (for about 50%) than L-isoleucine and L-leucine (Kennerknecht et al. 2002).

Export was identified as the most limiting step in L-isoleucine production (Morbach et al. 1996). Unfortunately the overexpression of *brnFE* (encoding BCAAs exporter) did not lead to the significant increase in L-isoleucine final concentration (Eggeling 2005).

1.6.3. Possible effect of the stringent response on amino acid overproduction

As it was already mentioned above often various amino acid auxotroph strains are used in amino acid overproduction. Amino acid auxotrophy can lead to stringent response induction as a response to diminished concentration of respective amino acid during cell growth or the stringent response could be evoked artificially by limited amino acid supply to the medium.

The stringent response is a pleiotropic physiological response of bacterial cells to amino acid or energy source limitation (Cashel et al. 1996). Many features of the stringent response are mediated by the accumulation of hyperphosphorylated guanosine nucleotide guanosine 5'-diphosphate 3'-diphosphate (ppGpp) in cell. *relA* (coding for ppGpp synthase) and *spoT* (coding for ppGpp hydrolase) regulated stringent mechanism has been described in gram negative bacteria *Escherichia coli* and *Salmonella typhimurium* (Cashel et al. 1996) andother bacteria.

The stringent response phenomenon in *Brevibacterium flavum* (nowdays *Corynebacterium glutamicum*) was first described by Ruklisha et al. (Ruklisha et al. 1993; 1995). It was showed that under conditions of L-threonine and L-methionine starvation, there was a sharp increase in the intracellular ppGpp concentration and a decrease in RNA synthesis rate. Later *relA/spoT* homologous gene, termed *rel,* that encodes a bifunctional enzyme with ppGpp synthase and ppGpp-degrading activities, has been described in *C. glutamicum* (Wehmeier et al. 1998).

The stringent response coordinates the global transcription pattern with the current growth conditions. Elevated ppGpp levels lead to an activation or repression of a variety of genes, resulting in a global reprogramming of the cell. The most prominent result is a growth arrest, accompanied by the downregulation of genes that are strongly expressed in fast growing cells e.g. those encoding ribosomal proteins, cell division and DNA replication proteins as well as a reduction of the amount of stable RNA (Cashel et al. 1996, Chatterji and Ojha 2001, Zhang et al. 2002).

Conversely, ppGpp appears to have a positive regulatory effect on the expression of some genes of amino acid biosynthesis. It has been shown that ppGpp mediates the increase in the expression of histidine biosynthetic operon in *Salmonella typhimurium* (Stephens et al. 1975) and is involved in the regulation of L-lysine biosynthesis in *E. coli* (Patte et al. 1980). It has been also reported that ppGpp is

required for the derepression of the genes of branched-chain amino acid synthesis in *E. coli* and *S. enterica* serovar typhimurium (Tedin and Norel 2001). Enzymes encoded by *ilv-leu* operon involved in the synthesis of branched chain amino acids were induced in a *rel*-dependent manner in *Bacillus subtilis* (Eymann et al. 2002).

Recently the changes in global gene expression in the stringent and relaxed *Corynebacterium* glutamicum mutants were investigated (Brockmann-Gretza et al. 2006). In total 357 genes were found to be transcribed differentially in Δ*rel* strain. The class of genes dependent on the functional *rel* gene included nitrogen metabolism genes (downregulated) and non-essential sigma factor gene *sigB* (upregulated). It was unusual to find that a large number of genes encoding ribosomal proteins were downregulated independently of *rel* gene in *C. glutamicum*. A set of genes encoding putative stress proteins and global transcriptional regulators were differentially regulated only in *rel*-deficient mutant. It was shown that there exist some notable differences of transcriptional reactions to the stringent response in *C. glutamicum* and those in *Escherichia coli* and *Bacillus subtilis*. These include *rel*-dependent regulation of the nitrogen metabolism genes and the *rel*-independent regulation of the genes encoding ribosomal proteins.

Considering the data the stringent response could be beneficial for L-valine synthesis mainly in two ways: (i) increase transcription activity of amino acid biosynthesis encoding genes, (ii) due to the decreased growth and respective increase in intracellular precursor concentration. The restriction of bacterial growth under the stringent response conditions (growth limitation by auxotrophic amino acids) has been previously found to increase NADPH availability and to increase L-lysine synthesis by homoserine auxotrophs of *C. glutamicum* (Ruklisha et al. 2001).

1.7. *C. glutamicum* constructs – L-valine overproducers

There exist amino acid overproducing strains obtained with several approaches: (i) conventional production strains generated by random mutation and selection of producing strains, (ii) strains with defined improvements in biosynthetic pathways (introduction of feedback resistant biosynthetic genes or the additional amplification of biosynthesis genes), (iii) strains with altered regulatory networks - e.g., L-lysine producing strains with minimized CO_2 formation, enhanced productivity at elevated temperatures, resistant to high osmotic pressure (Kelle et al. 2005).

Wild-type isolates of *C. glutamicum*, obtained by undirected mutagenesis and excreting L-valine have been described previously (Udaka and Kinoshita 1960, Nakayama et al. 1961).

The first generation of L-valine overproducing *C. glutamicum* strains were based on L-isoleucine auxotrophy due to *ilvA* deletion. Specific gene modifications in the wild-type *C. glutamicum* ATCC 13032 have been made:

(i) chromosomal deletion of the *ilvA* gene (coding for threonine dehydratase TD) (Sahm and Eggeling 1999). TD catalyses reaction where α-ketobutyrate (the precursor of L-isoleucine) is formed from L-threonine. Eliminated α-ketobutyrate level in Δ*ilvA* strains ensures more AHAS and pyruvate available for L-valine synthesis; strains with *ilvA* deletion are auxotrophic for L-isoleucine;

(ii) overexpression of the *ilvBN, ilvC and ilvD* genes on a plasmid (Sahm and Eggeling 1999) thus making more L-valine producing enzymes in cells available;

(iii) chromosomal deletion of the *panB* and *panC* genes (encoding ketopantoate hydroxymethyltransferase and D-pantothenate synthase respectively), to reduce D-pantothenate synthesis and CoA availability in the cells and therefore to reduce the pyruvate flow in the TCA (Radmacher et al. 2002). A second beneficial effect of the deletion of *panBC* is the increased availability of ketoisovalerate for synthesis of L-valine when less is used for production of D-pantothenate. Strains with *panBC* deletion became D-pantothenate auxotrophs. Further improvement of L-valine production was achieved by the construction of feedback resistant AHAS enzyme and its introduction into L-valine producing *C. glutamicum* strain (Elišáková et al. 2005).

Recently the second generation of *C. glutamicum* L-valine producers were constructed. This approach was based on the inactivation of pyruvate consuming enzymes and decoupling L-valine synthesis from growth. It involved the inactivation of pyruvate dehydrogenase complex and pyruvate:quinone oxidoreductase, and additional inactivation of phosphoglucose isomerase (in order to increase NADPH supply) and overexpression of the *ilvBNCE* genes on multicopy plasmid Blombach et al. 2007[b], 2008).

To avoid the presence of a plasmid in the cells of the C. g*lutamicum* producer, a new type of L-valine production strain was developed by promoter activity modulation (Holatko el. 2009). The activity of the promoters *ilvD* and *ilvE* genes was up-modulated and and the activity of the promoters of *ilv*A and *leuA* (encoding 2-isolproylmalate synthase) was down-modulated by site-directed mutagenesis. The

constructed strain was L-isoleucine bradytrophic and hence L-isoleucine supplementation to the growth medium is not necessary.

Nevertheless the successful strain construction, it is important to obtain knowledge about the synthesis enzyme regulation, and how the amino acid synthesis changes due to the physiological state of the cells. To achieve high amino acid synthesis productivity, it is important to investigate the synthesis physiology and biochemistry.

2. MATERIALS AND METHODS

2.1. Microorganisms

Bacterial strains used in this study are listed in Table 1.

Table 1. *C. glutamicum* ATCC 13032 recombinant strains used**,** Cm^R – resistance to chloramphenicol, Km^R – resistance to kanamycin

	Strain	Relevant characteristics	Reference
1.	Δ*ilvA* pECM3*ilvBNCD*	*ilvA* deleted, plasmid pECM3*ilvBNCD* Cm^R	Sahm and Eggeling 1999
2.	Δ*ilvA* pJC1*ilvBNCD*	*ilvA* deleted, plasmid pJC1*ilvBNCD* Km^R	Sahm and Eggeling 1999
3.	Δ*ilvA*Δ*panBC* pJC1*ilvBNCD*	*ilvA* and *panBC* deleted, plasmid pJC1*ilvBNCD* Km^R	Radmacher et al. 2002
4.	Δ*ilvA*Δ*panBilvN*M13 pECKA*ilvBNC*	*ilvA* and *panB* deleted, mutation in *ilvN*, plasmid pECKA*ilvBNC* Km^R	Elišáková et al. 2005
5.	Δ*ilvA*Δ*panBilvN*M13Δ*rel* pECKA*ilvBNC*	*ilvA, panB* and *rel* deleted, mutation in *ilvN*, plasmid pECKA*ilvBNC* Km^R	Denina et al. 2010

Besides, all strains had auxotrophy for biotin.

Strains No 1, 2 and 3 were supplied by Dr. L. Eggeling (Forschungszentrum Jülich, Germany), strains 4 and 5 were supplied by Dr. M. Patek (Academy of Sciences of the Czech Republic).

2.2. Cultivation of bacteria

2.2.1. Culture mediums

As complex medium for cultivation of *C. glutamicum* Brain Heart Infusion (BHI) medium and BHI agar plates were used. As defined minimal medium for *C. glutamicum* CGXII medium was used (Keilhauer et al., 1993). The composition of all media used is listed in table 2.

Table 2. Media used for cultivation of *C. glutamicum*. The composition is given for 1 liter.

Medium	Ingredients (per l)
BHI	37 g Brain Heart Infusion. Sterilization by autoclavation at 121°C for 20 minutes
CGXII	20 g $(NH_4)_2SO_4$, 5 g urea, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.25 g $MgSO_4*7H_2O$, 42 g MOPS, pH (KOH) 7.0. After autoclavation addition of: 10 mg $CaCl_2$, 10 mg $FeSO_4*7H_2O$, 3 mg deferoxeamine, 0.2 mg biotin, 1 ml trace element stock solution, 40 g glucose
Trace element stock solution	10 mg $MnSO_4*H_2O$, 1 mg $ZnSO_4*7H_2O$, 0.2 mg $CuSO_4*5H_2O$, 0.2 mg $NiCl_2*6H_2O$. Sterilization by filtration

2.2.2. Media supplements

Auxotrophy for L-isoleucine and D-pantothenate, due to *ilvA* or *panBC* deletion, requires the addition of L-isoleucine and D-pantothenate for cell culture growth in minimal medium. Therefore strains with *ilvA* deletion were supplemented with 1.143 mM L-isoleucine and strains with *panBC* or *panB* deletion with 0.75 μM D-pantothenate. Antibiotics supplemented to the growth medium of respective strains were chloramphenicol (5 mg l^{-1}) and kanamycin (50 mg l^{-1} for strains bearing plasmid pJC1*ilvBNCD* and 30 mg l^{-1} for strains bearing plasmid pECKA*ilvBNC)*.

2.2.3. Growth conditions

2.2.3.1. Precultivation

C. glutamicum strains were grown at 30°C in 500 ml baffled shake flasks on a rotary shaker (220 rpm). A standardized inoculation scheme was applied for cultivations: 50 ml BHI medium were inoculated from fresh BHI agar plates and grown for about 16 hours. Then the cells were harvested by centrifugation (4 000 x g, 4°C, 15 min), washed twice with 0.9% NaCl and used for inoculation either in bioreactor either in shake flasks.

2.2.3.2. Cultivation in bioreactor

The batch and fed-batch cultivations were performed in a 1.8 l working volume glass bioreactor (MBR, Switzerland), at 30°C, pH 7.0. The oxygen level was registered as dissolved oxygen partial pressure (pO_2) using a polarimetric oxygen electrode (Mettler Toledo, Switzerland) and adjusted by changing the volume of the ventilated air and the number of revolutions of the mixer. The pH was maintained at

7.0 by online measurement using a standard pH electrode (Mettler Toledo, Switzerland) and the automatic addition of 12 M NH_4OH. Foam development was prohibited by manual injection of small amounts (200 µl) of polypropylene glycol (Aldrich, Germany). When the glucose concentration in the medium fell to 6.0 ± 0.5 g l^{-1}, glucose supplementation was started in order to keep its concentration in the range 6.0-8.0 g l^{-1}.

2.2.3.3. Short-term experiments in shake flasks

Cells from precultivation were harvested by centrifugation (4 000 x g, 4°C, 15 min), washed twice with 0.9% NaCl and used for inoculation of CGXII medium to an optical density at a wavelength of 600 nm (OD_{600}) of 3. Cell culture was grown exponentially until reached maximum growth rate (µ=0.35-0.40 h^{-1}) and then again was harvested by centrifugation (4 000 x g, 4°C, 15 min) and washed twice with 0.9% NaCl. Depending on the condition to be investigated cells were treated differently:

- to cause L-isoleucine limitation, cultures were transferred to CGXII medium (depending on experiment):
    - lacking L-isoleucine
    - lacking L-isoleucine but supplemented with 10 – 100 mM L-valine.

  Control culture was resuspended in standard CGXII medium supplemented with 1.14 mM L-isoleucine. Incubation was carried out for 2.5-4 hours;
- to cause D-pantothenate limitation, cultures were transferred to CGXII medium lacking D-pantothenate or to CGXII medium supplemented with 0.38 µM D-pantothenate. Control culture was resuspended in standard CGXII medium with 0.75 µM D-pantothenate. Incubation was carried out for 3.5 hours;
- To cause oxygen limitation, cultures from exponential growth phase were transferred to shake flasks without baffles without centrifugation and washing step.

2.3. Estimation of biomass concentration

The growth of the cultures was followed by measuring the optical density of the cell culture at 600 nm (OD_{600}) using a Helios UV-Visible spectrophotometer (Thermo Spectronic, UK) after appropriate dilution to maintain apparent absorbance readings below 0.5. The biomass concentration was calculated from OD_{600} values using an

experimentally determined correlation factor of 0.3 g cell dry mass (cdm) l^{-1} for OD_{600}=1.

2.4. Amino acid estimation

Samples were directly taken from cultures and immediately processed by the silicone oil centrifugation method to produce a cytosolic fraction of metabolites (Klingenberg and Pfaff 1977). 100 µl of cell suspension was sedimented through 65 µl silicone oil (density 1.03g cm^3) into 30µl perchloric acid (20%,v/v) by centrifugation (13 000 x g, room tº, 2 min). Upper fraction was collected for determination of the external amino acid concentration. The tube was cut within the layer of oil, the bottom of tube was placed upside down in 0,5 ml Eppendorf tube and centrifuged (13 000 x g, room t°, 5 min). The cell pellet was resuspended in 45 µl H_2O together with 10 µl of silicone oil with density of 1.07 g cm^3 to absorb membrane material. After breaking up the cells by sonication (5 min, 42 kHz, Cole-Parmer 8890, USA), neutralizing the extract by addition of 25 µl buffer (5M KOH, 1M triethanolamine) and removing precipitated protein and cell wall material by centrifugation, the extract was used for intracellular amino acid analysis.

Due to the technical reasons two methods of high-pressure liquid chromatography (HPLC) were used:

1) The extracellular and intracellular amino acid concentration was determined by reversed-phase HPLC (Waters, USA) with fluorimetric detection (254 nm). In the pre- column step amino acids were derivatized with phenylisotiocyanate (PITC) to produce phenylthiocarbamyl (PTC) amino acids. Separation was carried out at 40°C on a PICO.TAG C18 column (particle size 5 µm, 300 x 3.9 cm, Waters, USA). The elution buffer A consisted of 6% acetonitrile in a phosphate buffer (pH 6.4), the elution buffer B consisted of 60% acetonitrile.

2) The extracellular and intracellular amino acid concentration was determined by reversed-phase HPLC (Agilent 1100, HP, USA) with fluorimetric detection (excitation at 340 nm and emission at 450 nm) after automatic precolumn derivatization with *ortho*-phthaldialdehyde. Separation was carried out at 40°C on a Hypersil AA-ODS column (particle size 5 µm, 200 x 2.1 mm; Agilent, USA). The elution buffer consisted of a polar phase (20 mM sodium acetate, pH 7.2 supplied with 0.018% TEA and 0.3% THF) and a nonpolar phase (20% 100 mM sodium

acetate, pH 7.2, 40% acetonitrile, 40% methanol). Quantification was performed with L-asparagine as an internal standard and by comparison of the sample peaks with an external standard. The intracellular concentration of amino acids was calculated taking into account the previously reported cell volume for *C. glutamicum* of 1.8 µl mg cdm^{-1} (Hermann and Kramer 1996).

2.5. Glucose estimation

Glucose concentration in the medium was estimated using Lane-Eynon titration method. It is based on reduced sugar reaction with copper sulphate.

Solutions:

Solution I: 10 g $CuSO_4$, 0.04 g metilenblue (per 1 l)

Solution II: 50 g KNa tartrate, 75 g NaOH, 5 g $K_4[Fe(CN)_6]$ (per1 l).

In conical flask 5 ml of solution I was boiled together with 5 ml of solution II for 30 sec, than the mixture was titrated with analyses until the solution changed colour (blue → white). Glucose concentration was estimated after equation:

Glucose % = ((Factor) x (analyses dilution factor)/ ml of analyses for titrating

Factor:

5 ml solution I and 5 ml solution II was titrated with glucose standart solution (0.1 g glucose dissolved in 100 ml H_2O).

Factor = (g glucose) x (titrated ml)

2.6. Total reducing activity (TRA) estimation

The total reducing activity was estimated as the specific rate of 2.3.5-triphenyltetrazolium chloride reduction by intact cells (Jambor et al. 1959).

2 ml of cell culture was centrifuged (13 000 x g, room tº, 3 min), washed with phosphate buffer (70 mM KH_2PO_4, 70 mM Na_2HPO_4, 10 mM KCl, 5 mM $MgCl_2$, pH 7.0), and resuspended in the same buffer to reach final biomass concentration 1.3 g l^{-1}. 1 ml of 0.1% TTC solution in the phosphate buffer and 1 ml of cell suspension were poured into different chambers of a Tunberg-type tube not allowing solutions to mix. The solutions was degassed by vacuum pump and then quickly mixed under anaerobic conditions to start the reaction. The reaction solution was incubated at 30ºC for 1h. The reaction was terminated by addition of 3 ml of acetic acid and the formed

formazan was extracted in 10 ml of toluol (2h, room tº, periodically mixing). The extracted formazan in toluol fraction was measured at 500 nm. The calibration was done with pure TTC. TRA was calculated as the specific rate of formation of the reduced form of TTC (mmol TTC-reduced g dw^{-1} h^{-1}).

2.7. Cellular respiration activity

Cellular respiration activity was characterised by the cellular oxygen consumption rate. The cellular oxygen consumption rate (Q_{O2}) was measured with a Clark-type oxygen electrode (Radiometer, Denmark). The samples of the cell culture from bioreactor or flasks were diluted with the same growth medium up to 1 mg cdm ml^{-1} and transferred to the electrode chamber. To measure the cyanide-resistant respiration ($Q_{O2\ CN\text{-}resistant}$), which characterizes oxygen consumption uncoupled from ATP synthesis, 1 mM KCN was supplemented to the cell culture sample (Toma et al. 1984). The cyanide-sensitive respiration ($Q_{O2\ CN\text{-}sensitive}$), which characterizes oxygen consumption coupled with ATP synthesis, was calculated as the difference between $Q_{O2\ summary}$ and $Q_{O2\ CN\text{-}resistant}$.

The $Q_{O2\ summary}$ and $Q_{O2\ CN\text{-}sensitive}$ was expressed in nmol mg cdm^{-1} min^{-1} taking into account that 1 unit corresponds to 0.75 nmol O_2 ml^{-1}. $Q_{O2\ CN\text{-}resistant}$ was expressed as the percentage of summary respiration.

2.8. Experimental data calculations

The cellular growth rate (μ), the cell specific rate of L-valine synthesis (q_P), the specific rate of glucose consumption (q_S), experimental yields of cell mass and L-valine on glucose ($Y_{X/S\ exp}$ and $Y_{P/S\ exp,}$ respectively) and the productivity (Q_P) were calculated using the experimental data.

The equations were as follows:

$$\mu=2.3(\lg X_2-\lg X_1)/t_2-t_1$$

X_1, X_2 – biomass concentration in respective $time_1$ and $time_2$

$$q_P=\Delta P/X_{average}*h$$

ΔP – difference between product concentrations in respective time points
$X_{average}$– average biomass concentration in respective time
h - time

$q_S = \Delta Glu / X_{average} * h$

ΔGlu – difference between glucose concentrations in respective time points
$X_{average}$ – average biomass concentration in respective time
h - time

$Q_P = X * q_S * Y_{P/S}$

X – biomass concentration
q_S - the specific rate of glucose consumption
$Y_{P/S}$ - L-valine experimental yield from glucose

$Y_{P/S} = \Delta P / \Delta S$

ΔP - difference between product concentrations in respective time points
ΔS - difference between sugar concentrations in respective time points

$Y_{X/S} = \Delta X / \Delta S$

ΔX – difference between biomass concentrations in respective time points
ΔS - difference between sugar concentrations in respective time points

2.9. Estimation of L-valine synthesis enzyme activities

2.9.1. Preparation of the crude extracts

The cells were harvested by centrifugation (4 000 x g, 4°C, 15 min). The cell pellet was washed twice with 0.2% KCl at 4°C. The cells were then resuspended in 100 mM potassium phosphate buffer (pH 7.3) with 0.5 mM dithiothreitol and 20% (v v^{-1}) glycerol.

Cell-free extracts were obtained by sonication (UPs200, Hielscher Ultrasonics, GmbH, Germany) of the cell suspension (50 kHz, 8 min of 0.5 s and 0.5 s space out). Cellular debris was removed by centrifugation (10 000 x g, 4°C, 30 min) and the supernatant was kept on ice until the enzyme determination or stored at -20°C.

2.9.2. Quantification of proteins

Protein concentrations in the cell-free extracts were determined spectrophotometrically (Helios Gamma, UK) according to Lowry et al. (1951).

Reagents:

Reagent A: 2% Na_2CO_3 solution in 0.1 N NaOH

Reagent B: 0.5% $CuSO_4 * 5H_2O$ solution in 1% $Na_3C_6H_5O_7 * 2\ H_2O$

Reagent C: alkaline copper solution (50:1): mix 50 ml o Reagent A with 1 ml of Reagent B

Folin reagent

To the 1 ml of protein sample 5 ml of reagent C were added and reaction mixture was incubated for 10 minutes at room t°. Then 0.5 ml of Folin reagent was added, reaction mixture was mixed rapidly and incubated for another 30 minutes at room t°. Then the absorbance at 540 nm was measured and the protein concentration was determined using the standard curve. For a standard curve estimation bovine serum albumin (BSA) was used.

2.9.3. AHAS activity assay

The AHAS activity in the cell-free extracts was determined by a method based on the enzymatic conversion of pyruvate to acetoin through α-acetolactate as modified by Leyval et al. (2003). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), 50 mM sodium pyruvate, 10 mM $MgCl_2$, 100 μM thiamine pyrophosphate and 100 μM flavin adenine dinucleotide. The reaction was initiated by the addition of 0.1 ml of the crude extract to 0.9 ml of the enzyme assay mixture. The reaction mixture was incubated at 37°C for 20 min and the reaction was terminated by acidification (addition of 0.1 ml of 50% H_2SO_4). The reaction mixture was then incubated for an additional 30 min at 37°C to allow the α-acetolactate to be converted into acetoin. The acetoin formed was quantified using the Voges-Proskauer method (1945). The absorbance of the reddish reaction mixture was measured spectrophotometrically at 535 nm. The specific AHAS activity was expressed in nmol of α-acetolactate formed per mg protein per min.

2.9.4. AHAIR activity assay

AHAIR activity was determined by the method developed by Arfin and Umbarger (1969) and modified by Leyval et al. (2003). The reaction mixture (0.99 ml) consisted of 100 mM potassium phosphate buffer (pH 7.3), 10 mM α-acetolactate, 3 mM $MgCl_2$ and 0.05ml crude extract. The AHAIR reaction was initiated by adding of 0.01 ml of 10 mM NADPH to the reaction mixture and was performed at 30 °C. NADPH consumption was monitored at 340 nm**.** The specific AHAIR activity was expressed in nmol of NADPH oxidized per mg protein per min.

Synthesis of α-acetolactate (Muniglia 2001):

The synthesis was performed under N_2 atmosphere. 40 mg ethyl-2-acetoxy-2-methylacetolactate was dissolved in 4 ml 0.1 M NaOH (previously deoxygenated with N_2). The reaction mixture was continuously agitated for 30 minutes at room tº. All the ethyl-2-acetoxy-2-methylacetolactate is converted into α-acetolactate. Due to the instability of this product, the solution was used immediately. Ethanol and acetate have no influence on the AHAIR activity, for this reason α-acetolactate was not purified.

2.9.5. DHAD activity assay

The DHAD activity was assayed using the method of Flint et al. (1993). The method is based on the formation of the 2,4-dinitrophenylhydrazone from the keto acid product of the enzymatic reaction. The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$ and 10 mM DL-α,β-dihydroxyisovalerate. The reaction was initiated by the addition of 0.1 ml of the crude extract to 0.9 ml of the reaction mixture. The reaction run at 37°C for 10 min and was terminated by the addition of 0.1 ml of 2,4-dinitrophenylhydrazine saturated in 2N HCl. This hydrazine reacted with α-ketoisovalerate, product of the DHAD reaction, to give α-ketoisovalerate-dinitrophenylhydrazone. The concentration of this product was then determined at 540 nm. The specific DHAD activity was expressed in nmol of α-ketoisovalerate formed per mg protein per min.

DL-α,β-dihydroxyisovalerate was synthesized in Institute of Organic Chemistry (Latvia) according to reported method (Cioffi et al., 1980).

2.9.6. Transaminase B activity assay

The TmB assay is based on the determination of α-ketoglutarate produced by the amination of α-ketoisovalerate (TmB substrate) using L-glutamate as an amino group donor. The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 40 mM L-glutamate, 4 mM α-ketoisovalerate and 0.25 mM pyridoxal 5'-phosphate monohydrate. The reaction was initiated by the addition of 0.1 ml cell-free extract to 0.9 ml of reaction mixture. The reaction was performed at 30ºC for 10 min, and then terminated by the addition of 0.1 ml of 20% H_3PO_4**.** The sample was centrifuged (13 000 x g, 4^{o}C, 5 min) and the supernatant neutralized by the addition of 5 M KOH

solution in 1 M triethanolamine**.** In the next step, α-ketoglutarate, produced in the TmB reaction, was quantified enzymatically with the help of $NADP^+$-specific L-glutamate dehydrogenase. The TmB specific activity was expressed in nmol of α-ketoglutarate, formed per minute per mg of protein.

2.10. Assay of central metabolism enzymes activity

The crude extract preparation and protein quantification was done as described above in chapters 2.9.1 and 2.9.2. The crude extract preparation for aconitase determination is described in chapter 2.10.3. All enzymes were assayed by spectrophotometric measurement (Helios Gamma, UK) of variation in $NADH_2$ or NADPH concentrations at 340 nm (e = 6.22 M^{-1} cm^{-1}). One unit of enzyme activity was defined here as the amount of an enzyme which converted 1 nmol of the substrate per min per mg of protein. All enzymes (with exception of aconitase) were assayed at 30 ºC and pH 7.5.

2.10.1. G-6-PDH activity assay

For the glucose-6-phosphate dehydrogenase assay modified method of Moritz et al. (2002) was used. It is based on G-6-PDH ability to oxidize glucose-6-phosphate using $NADP^+$ as cofactor. The assay system (1 ml) for determination of G-6-PDH activity contained 50 mM Tris/Cl pH 7.5, 40 mM $MgCl_2$, 0.5 mM $NADP^+$ and the crude extract. The reaction was initiated by the addition of 2 mM glucose-6-phosphate.

2.10.2. 6-PFK activity assay

6-phosphofructokinase was assayed by coupling the formation of fructose- 1,6-bisphosphate to the oxidation of NADH via the enzymes fructose bisphosphate aldolase, triose phosphate isomerase and glycerol-3-phosphate dehydrogenase according to the method of Sugimoto and Shiio (1987). The reaction mixture contained 0.1 M Tris-HCl, pH 7.5, 0.2 mM NADH, 10 mM $MgCl_2$, 4.0 mM fructose-6-phosphate, 1 mM ATP, 40 μg aldolase, 20 μg of the mixture α-glycerophosphatdehydrogenase and triose-phosphate isomerase. The reaction was started by the addition of the crude extract.

2.10.3. AC activity assay

The crude extract preparation was done according to the method of Krug et al. (2005). Cells (25 ml) were harvested with 25 g of crushed ice (precooled to –20 °C) by centrifugation (4 000 x g, 4°C, 15 min). The cell pellet was resuspended in Tris-HCl buffer (90 mM, pH 8.0) and the cells were mechanically disrupted by 3 min 1000 x g bead beating with 2 g of silica beads (diameter 150-212 microns, Sigma, Germany). After centrifugation (10 000 x g, 4°C, 30 min), the supernatant was immediately used for the enzyme assay.

Aconitase activity was assayed by following the formation of *cis*-aconitate from isocitrate (Henson and Cleland 1967) at room t°. The assay mixture contained 900 μl 90 mM Tris-HCl, pH 8.0, containing 20 mM DL-trisodium isocitrate. The reaction was started by the addition of 100 μl crude extract and *cis*-aconitate formation was determined by measuring the absorbance increase at 240 nm. An extinction coefficient for *cis*-aconitate of 3.6 mM^{-1} cm^{-1} at 240 nm was used. One unit of activity corresponded to 1 μmol isocitrate converted to *cis*-aconitate per min.

2.10.4. LDH activity assay

The method is based on LDH ability to convert pyruvate in lactate in presence of NADH. The assay system for the determination of LDH activity contained 50 mM Tris-HCl, 2 mM sodium pyruvate and 0.2 mM NADH. The reaction was initiated by the addition of the crude extract.

2.10.5. NADH dehydrogenase assay

NADH dehydrogenase was assayed by using a reaction mixture containing Tris-HCl buffer (100 mM), $MnSO_4$ (5 mM), and $NADH_2$ (0.6 mM) (Cocaign-Bousquet et al. 1996). The reaction was initiated by the addition of the crude extract. .

2.11. Assay of intracellular compounds

The samples for determination of intracellular metabolite concentrations were treated with modified cold methanol quenching method described by de Konig and van Dam (1992). 10 ml of cell culture was quickly dropped into the centrifuge tube with 20 ml 60% methanol supplemented with 50 mM HEPES (pH 7.5) (precooled to –20°C) and stored for 20 min. After that cells were centrifuged (5000 x g, –10°C, 15 min) and further used for pyruvate and NADPH extraction with respective methods.

2.11.1. Pyruvate enzymatic estimation

The extraction of pyruvate was performed in perchloric acid (pH 1.0, 0°C, 25 min) (Weibel et al. 1974). Then the extracts were centrifuged (10 000 x g, 4°C, 15 min) and neutralized to pH 7.0 with 5N KOH /0.1 N TEA. Pyruvate concentration in extracts was determined enzimatically using L-lactic dehydrogenase (EC 1.1.1.27). Reaction mixture contained 750 mM TEA, 7.5 mM EDTA, pH 7.6, 300 mM NADH and 3 U/ml LDH. Absorbance was estimated at 340 nm, room t°.

2.11.2. NADPH enzymatic estimation

The extraction of NADPH was performed by KOH (pH 12.5, 50°C, 12 min). Then extracts were rapidly cooled (ice-water, 10 min) and centrifuged (10 000 x g, 4°C, 15 min) to remove cell debris. Extracts were neutralized to pH 7.0 with 0.77 N HCl.

NADPH concentration in extracts was determined enzimatically using glutamate dehydrogenase (E.C. 1.4.1.3) as described by Klingenberg (1974). The reaction mixture contained 200 mM TrisHCl buffer pH 7.5, 30 mM α-oxoglutaric acid, 2 M NH_4Cl, 1.5 U/ml $NADP^+$-specific glutamate dehydrogenase. Absorbance was estimated at 340 nm, room t°.

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2.11.3. ppGpp estimation

Guanosine 5'-diphosphate 3'-diphosphate (ppGpp) was extracted from the cells with 0.2 M KOH as described by Zhang et al. (2002). 60 ml of culture were mixed with 6 ml of 2% formaldehyde and kept on ice for 25 min. The cells were sedimented by centrifugation (4500 x g, 4°C, 15 min) and lysed by resuspension in 0.5 ml of 0.2 M KOH with vigorous mixing. After 25 min on ice, the lysates were neutralized with 5 μl of concentrated (85%) phosphoric acid before 0.5 ml HPLC buffer was added. The cell debris was removed by centrifugation (10 000 x g, 4 °C, 20 min) and the supernatant was used for ppGpp concentration measurement.

Nucleotides in cell extracts were separated by isocratic ion exchange HPLC (Waters 501) with a 4.6 mm x 250 mm Hypersil Sax 5 μm column, using a 0.03 M potassium phosphate buffer (pH 3.4), supplemented with 14% acetonitrile and 0.01 M tetrabutylammonium phosphate, as the mobile phase. ppGpp was quantified by

measuring the absorbance at 254 nm using a Tunable Absorbance Detector (Waters 486, Waters, USA).

2.11.4. Nucleotide (ATP, ADP, AMP) estimation

Nucleotides were extracted from the cells with perchloric acid as described by Weibel et al. (1974). 8 ml of cell culture were dropped in centrifuge tube with 2 ml pre-cooled 35% $HClO_4$, mixed rapidly and kept on ice for 25 min. The cell debris was removed by centrifugation (10 000 x g, 4°C, 10 min). 3 ml of supernatant was neutralized to pH 7.0-7.4 with gradual addition of pre-cooled 2N $KHCO_3$ (10 min, 20°C). The sediment was removed by centrifugation (10 000 x g, 4°C, 20 min) and the supernatant was used for nucleotide estimation.

The nucleotides were separated by isocratic reversed-phase HPLC (Waters 501, USA) with a 4.6mm x 250 mm Adsorbosphere Nucleotide–Nucleoside 5µm column () (Alltech, USA). The mobile phase used for chromatography was 0.2 M potassium phosphate buffer, pH 6.05. Nucleotides were quantified by measuring the absorbance at 254 nm using a Tunable Absorbance Detector (Waters 486, Waters, USA).

2.12. Chemicals

The synthesis of α-acetolactate, which was used for the AHAIR assay, was carried out according to the reported method (Muniglia 2001). DL-α,β-dihydroxyisovalerate, used for the DHAD assay, was synthesized according the method described by Cioffi et al. (1980) in the Latvian Institute of Organic Synthesis (Riga, Latvia). All other chemicals were of analytical grade and commercially available.

2.13. Data processing

To determine the kinetic parameters at least 3 runs were performed with 3 replicates for each estimate of the mean. Standard deviation of the mean was calculated using Microsoft Excel and is shown as error bars in figures.

3. RESULTS

3.1. Characterisation of growth and L-valine synthesis by various *C. glutamicum* recombinant strains

The strains used in this study differed with genetic alterations in the chromosome (*ilvA* or simultaneous *ilvA* and *panBC* gene deletion) and had L-valine synthesis genes overexpressed on different plasmids (pJC1*ilvBNCD* or pECM3*ilvBNCD*). The cell specific growth rate (μ), specific rate of L-valine synthesis (q_P), L-valine synthesis enzyme AHAS activity and intracellular L-valine concentration were chosen as parameters to characterise the growth and L-valine synthesis activity of strains.

During the batch cultivations the highest growth rate was reached by *C. glutamicum* Δ*ilvA* (pECM3*ilvBNCD*): μ_{max} of this strain was 1.9- and 1.5-fold higher than values reached by the cells of *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) and *C. glutamicum* Δ*ilvA* (pJC1*ilvBNCD*), respectively (Fig 5A). Also the maximum biomass was reached by strain Δ*ilvA* (pECM3*ilvBNCD*) (Fig. 5B). In contrast, maximum L-valine synthesis activity was reached by *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*): q_{Pmax} of this strain was 5.7- and 1.4- fold higher than values reached by cells of *C. glutamicum* Δ*ilvA* (pECM3*ilvBNCD*) and *C. glutamicum* Δ*ilvA* (pJC1*ilvBNCD*), respectively (Fig. 5C). Both strains *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) and *C. glutamicum* Δ*ilvA* (pJC1*ilvBNCD*) exhibited high AHAS activity during the batch cultivations in contrast to strain *C. glutamicum* Δ*ilvA* (pECM3*ilvBNCD*), where AHAS activity was quite low and remained stable during the cultivation (Fig. 5D). Intracellular L-valine concentration in the cells of *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) was much higher compared to values estimated for other strains and reached up to 250 mM (Fig. 5E). Note that wild type *C. glutamicum* ATCC 13032 produced only about 1mM L-valine (data not shown).

Thus it was shown that variations in strain genotype influenced the strain growth and L-valine synthesis capability. The best L-valine production was achieved by strains *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) and *C. glutamicum* Δ*ilvA* (pJC1*ilvBNCD*). These strains had a comparatively low cellular growth rate, comparing with strain *C. glutamicum* Δ*ilvA* (pECM3*ilvBNCD*), but an increased

AHAS and L-valine synthesis rate. Although the only difference between *C. glutamicum* Δ*ilvA* (pECM3*ilvBNCD*) and *C. glutamicum* Δ*ilvA* (pJC1*ilvBNCD*) was the basis vector, it was obvious that it influenced L-valine synthesis capability (Fig. 5F). Considering the results, strains *C. glutamicum* Δ*ilvA*Δ*panBC* (pJC1*ilvBNCD*) and *C. glutamicum* Δ*ilvA* (pJC1*ilvBNCD*) were chosen as most prospective for use in further experiments. Strain *C. glutamicum* Δ*ilvA* (pECM3*ilvBNCD*) was excluded from further study.

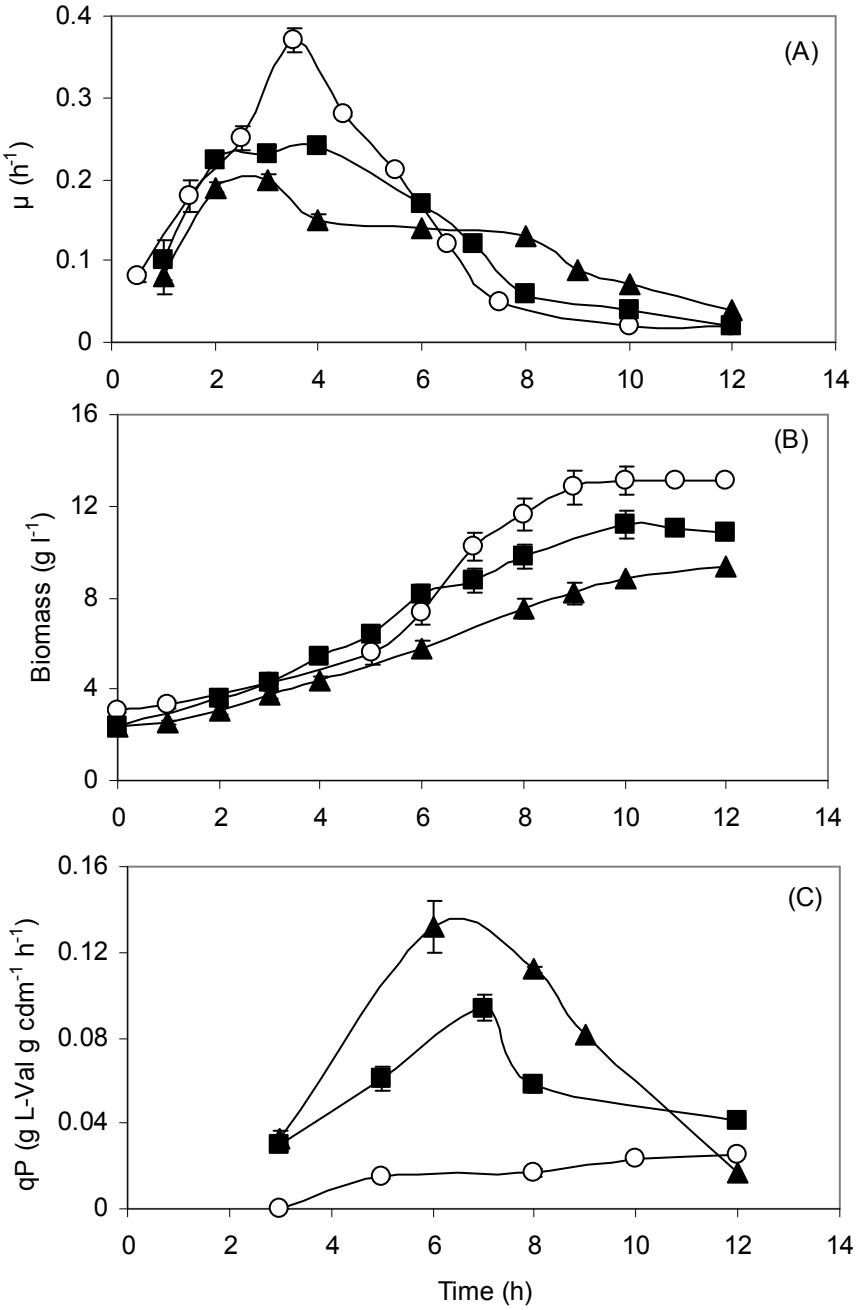

Fig. 5. Growth rate (**A**), biomass concentration (**B**) and L-valine synthesis rate (**C**) in batch cultivations of (▲) *C. glutamicum ΔilvA ΔpanBC* (pJC1*ilvBNCD*), (■) *C. glutamicum ΔilvA* (pJC1*ilvBNCD*) and (O) *C. glutamicum ΔilvA* (pECM3*ilvBNCD*).

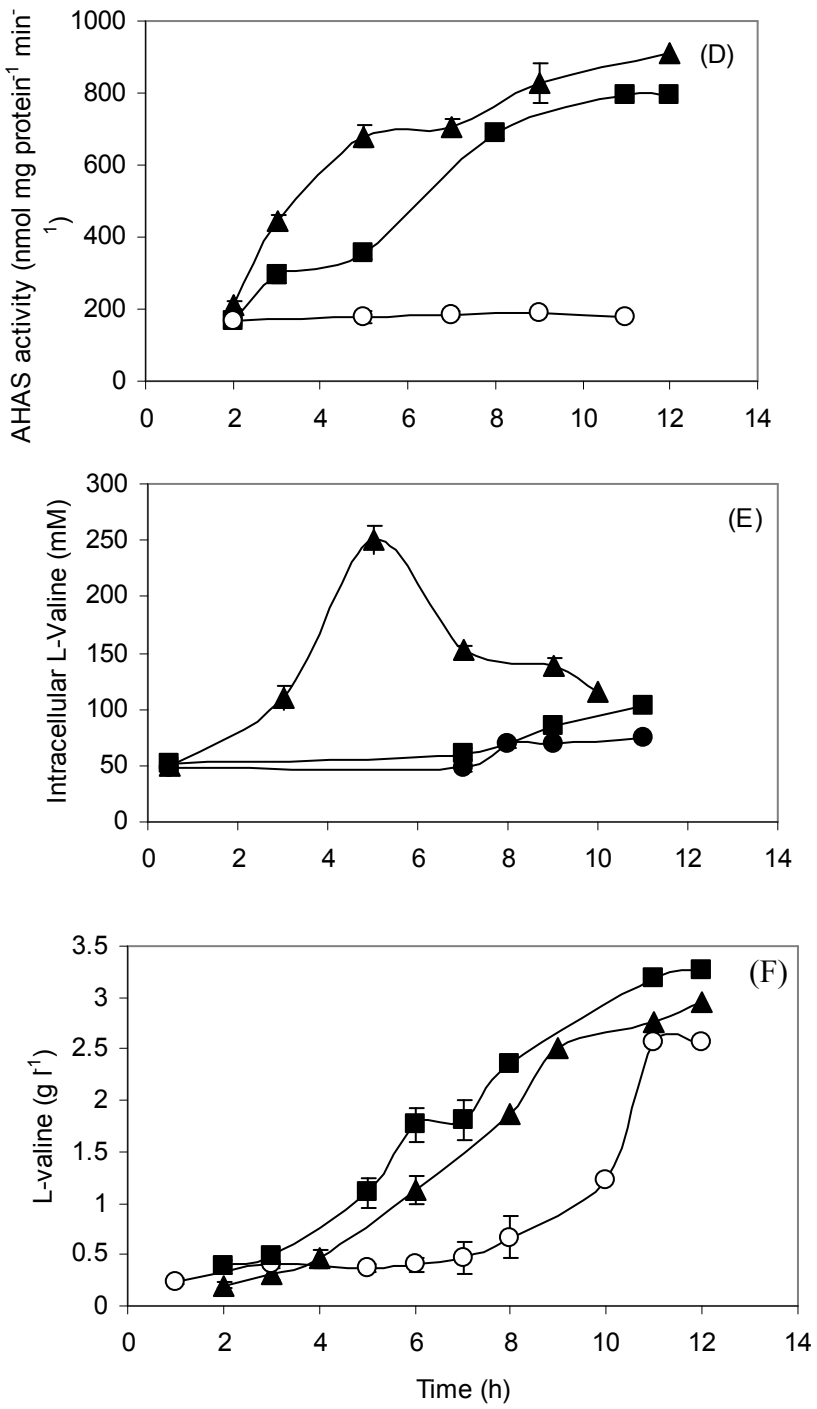

Fig. 5 (continued). Activity of acetoxydroxyacid synthase (AHAS) (**D**), intracellular concentration of L-valine (**E**) and **(F)** extracellular concentration of L-valine in batch cultivations of (▲) *C. glutamicum ΔilvA ΔpanBC* (pJC1*ilvBNCD*), (■) *C. glutamicum ΔilvA* (pJC1*ilvBNCD*) and (O) *C. glutamicum ΔilvA* (pECM3*ilvBNCD*).

It was observed that AHAS activity and L-valine synthesis activity increased after the cellular growth rate started to decrease below maximum in all studied strains. Therefore it was suggested that a transition in the cellular growth rate to below

maximum could be the optimum physiological state of cell culture for an increase in L-valine synthesis activity.

3.2. Identification of optimal cellular physiology for L-valine overproduction

In order to elucidate the optimum physiological state of cell culture for L-valine synthesis, parameters characterising the bacterial growth and L-valine synthesis were analysed during the batch cultivations of strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*).

A correlation between the changes in the cellular growth rate and the cellular total reducing activity (TRA, characterising electron transport chain activity and estimated as the specific rate of 2.3.5-triphenyltetrazolium chloride reduction by intact cells) was observed. The decrease in TRA correlated with the decrease in cyanide-sensitive oxygen consumption, characterising ATP-synthesis-coupled respiration of cells (Fig. 6). The decrease in cellular growth rate also was related to a slight decrease in the cell energy charge (showing the ratio between adenylate cofactors in cell). At the maximal growth rate (μ=0.20 h^{-1}) EC value was 0.95, but it decreased to 0.67 after the decrease in cellular growth rate (μ=0.14 h^{-1}) (data not shown). The decrease in cellular growth rate correlated with decrease in biomass experimental yield as well (Fig.7).

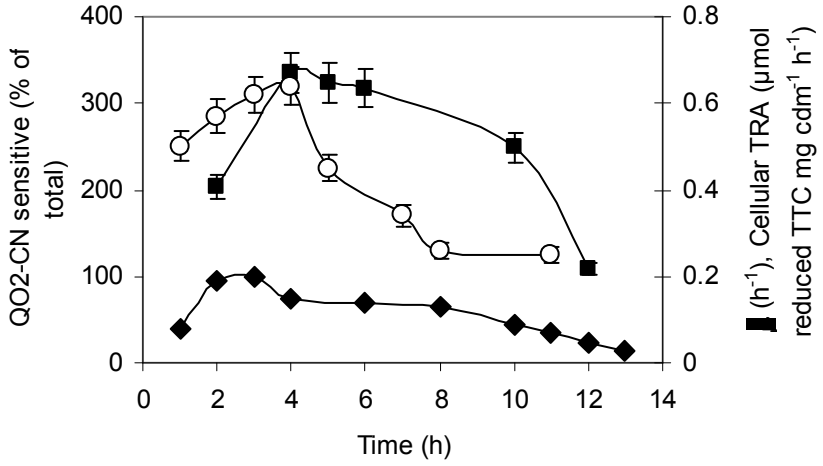

Fig. 6. Changes in cyanide-sensitive respiration ($QO2_{CN\text{-}sensitive}$) (o), cellular growth rate (μ) (♦) and cell total reducing activity (TRA) (■) in *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) during batch cultivation.

These results showed that cellular growth was characterised by high activity of the electron transport chain and a high cell energetic state. These parameters reflected high cell demand for ATP synthesis.

After the cellular growth rate downregulation and the decrease in energy generation there was observed an increase in L-valine synthesis. The activity of AHAS increased sharply and remained high during the cultivation. Also L-valine synthesis rate and L-valine experimental yield ($Y_{P/S}$) reached maximum after the cellular growth rate downregulation: q_P was 0.04 g L-val g $cdm^{-1}h^{-1}$ at μ_{max} 0.20 h^{-1} but q_{Pmax} 0.13 g L-val g $cdm^{-1}h^{-1}$ was reached when μ decreased to 0.12 h^{-1} (Fig. 7). Therefore it was considered that the decrease in cellular growth rate and respectively the decrease in energy generation in cells was favourable to increase L-valine synthesis activity.

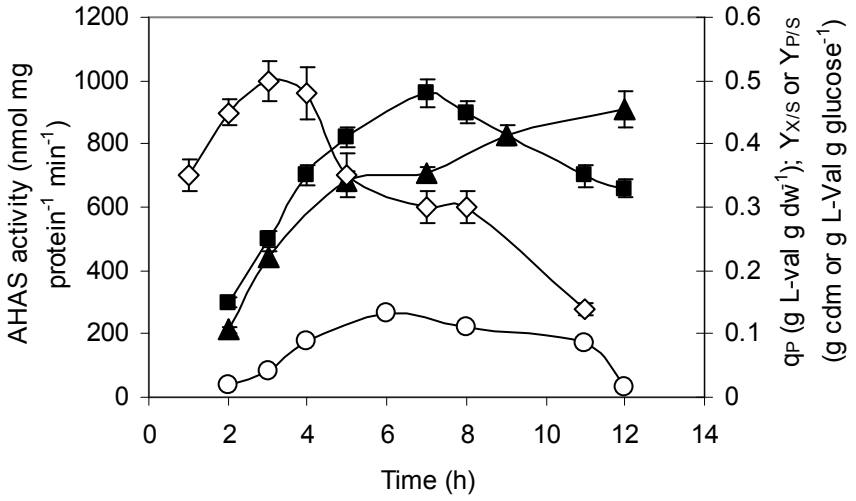

Fig. 7. Changes in acetohydroxyacid synthase (AHAS) activity (▲) and L-valine synthesis rate (q_P) (o), biomass ($Y_{X/S}$) (◊) and L-valine ($Y_{P/S}$) experimental yield (■) in *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) during batch cultivation.

However L-valine synthesis activity decreased when the cellular growth rate fell below 0.05 h^{-1}. Therefore it was assumed that the cellular growth rate should be maintained below μ_{max} but above 0.05 h^{-1} to maintain optimum L-valine synthesis rate in cells.

3.3. Identification of parameters of intracellular biochemistry enhancing L-valine overproduction

The experimental results described above showed that the transition in cellular growth rate to below maximum during batch cultivation was optimum physiological condition of cell cultures of *C. glutamicum* recombinants for L-valine overproduction. It was suggested that the increase in L-valine overproduction by recombinant strains was connected with the cellular growth rate downregulation associated changes in cell metabolism.

In order to explain the increase in L-valine overproduction by *C. glutamicum* recombinants during the transition in the cellular growth rate below maximum (μ below 0.25 ± 0.05 h^{-1}), the relationship between the variations in cellular growth rate, the activity of key enzymes of central metabolism and L-valine synthesis, and in amino acid precursor concentration in cells during fed-batch cultivations of strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNC*) was investigated.

The phase of growth when the cellular growth rate started to decrease was characterised with a rapid increase in AHAS activity. The increase in AHAS was related to the increase in the intracellular concentration of pyruvate (Fig. 8A,B). The decrease in aconitase (AC) activity indicated to decrease in TCA activity. There was also observed a correlation between the increase in L-valine synthesis rate and the increased activity of G-6-PDH. However a correlation between changes in L-valine synthesis activity, intracellular concentration of NADPH and the activity of 6-PFK were not observed (Fig. 8C).

The increase in AHAS activity was explained by the increased level of intracellular pyruvate – substrate for AHAS. The increase in pyruvate could result from (i) decreased pyruvate flow into TCA, (ii) the decrease in the cellular growth rate and consequently decreased pyruvate utilisation for biomass synthesis. The positive correlation between the increase in L-valine synthesis rate and the activity of G-6-PDH might reflect an increase in NADPH generation in response to its increased demand for L-valine synthesis. Thus it was assumed that the parameters enhancing the increase in L-valine synthesis by growth-restrained *C. glutamicum* recombinant cells were (i) increase in intracellular concentration of pyruvate, (ii) increase in AHAS activity, (iii) increase in G-6-PDH activity.

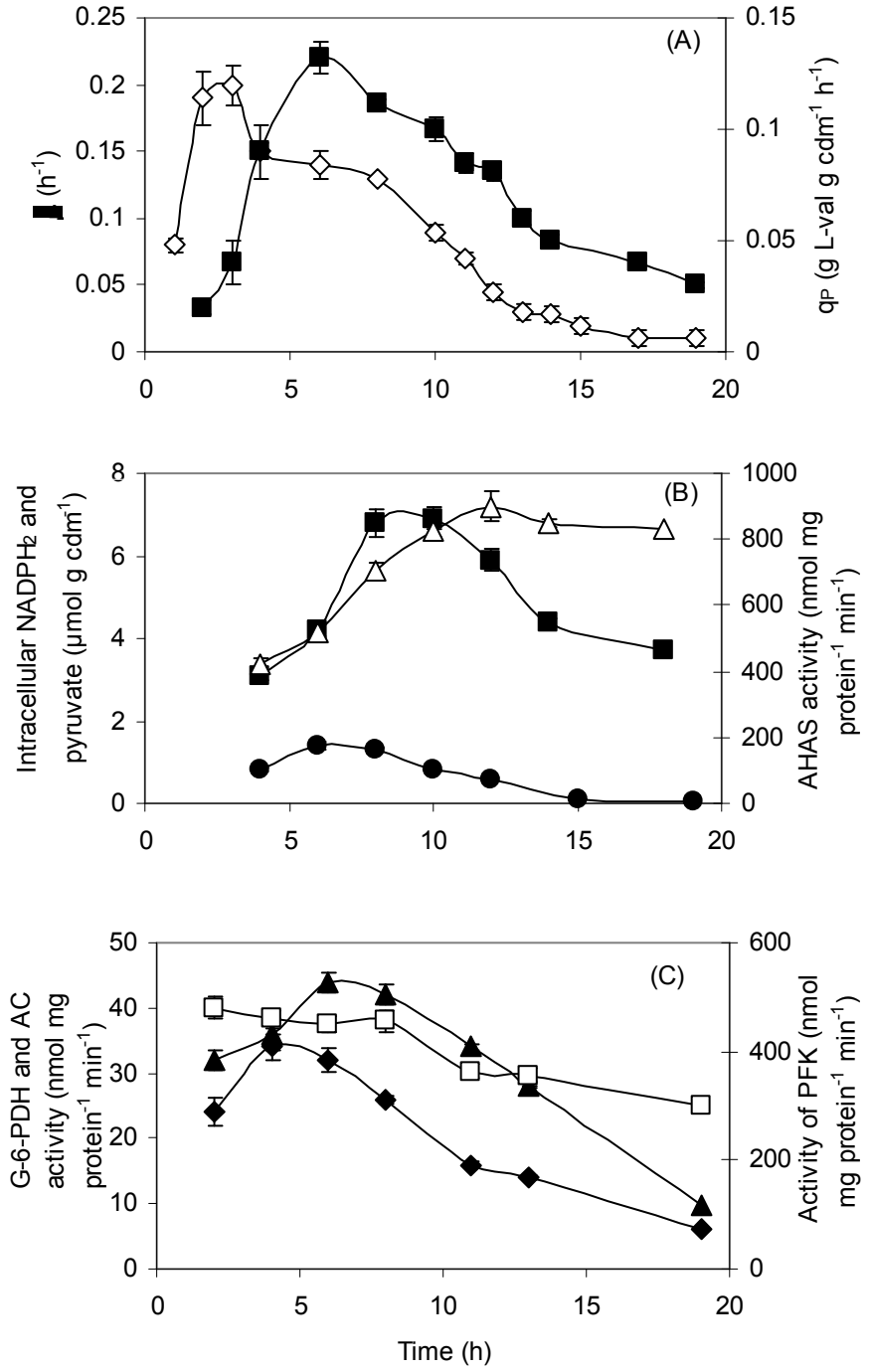

Fig. 8. Variations in parameters of physiology and biochemistry of *C. glutamicum ΔilvA ΔpanBC* (pJC1*ilvBNCD*) during fed-batch cultivations. **(A)**: cellular growth rate (μ) (◊), the cell specific L-valine synthesis rate (qP) (■), **(B)** intracellular concentrations of pyruvate (■) and NADPH (●), acetohydroxyacid synthase (AHAS) activity (Δ); **(C):** the activity of glucose-6-phosphate dehydrogenase (G-6-PDH) (▲), aconitase (AC) (◆) and 6-phosphofructokinase (PFK) (□).

However, a similar relation between AHAS activity and intracellular pyruvate concentration was not observed in strain *C. glutamicum* Δ*ilvA* Δ*panB* ilv*N*M13 (pECKA*ilvBNC*) (this strain was used to study the effect of L-isoleucine limitation and is described in details in chapter 3.5). Although the AHAS activity increased, after the cellular growth rate decreased below maximum, and the enzyme activity remained high during the cultivation, as it was shown experiments with other strains, the intracellular pyruvate concentration decreased during the growth (Fig. 9). Similarly there was not observed an increase in G-6-PDH activity. Only minor changes in intracellular NADPH concentration were seen. Therefore it was suggested that AHAS activity could be the main regulatory parameter in L-valine synthesis, but the role of precursor should be further investigated. Besides, it was concluded that G-6-PDH activity actually resembles NADPH utilisation for L-valine synthesis.

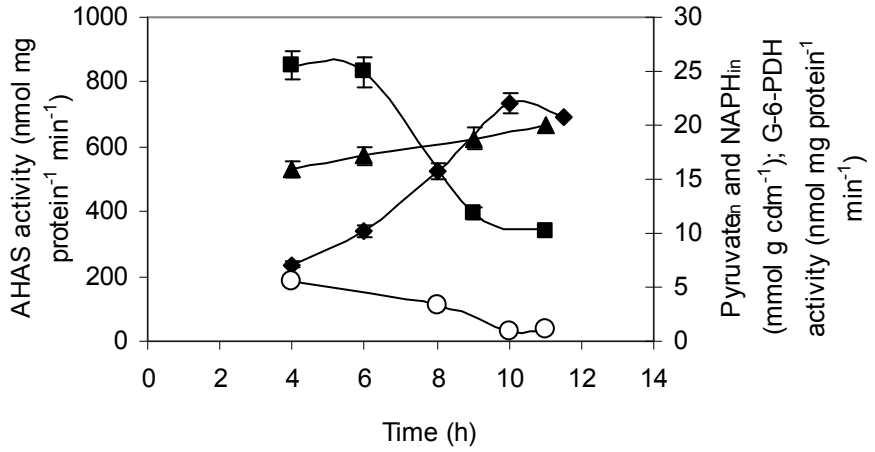

Fig. 9. Kinetics of AHAS (◆) and G-6-PDH (○) activity, concentration of pyruvate (■) and NADPH (▲) in cells during the batch cultivation of *C. glutamicum* Δ*ilvA* Δ*panBilvN*M13 (pECKA*ilvBNC*).

3.4. Effect of the increase in pyruvate concentration on L-valine synthesis

As it was shown above, the increase in the intracellular concentration of pyruvate directly correlated with the increase in AHAS activity in strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*), but not in strain *C. glutamicum* Δ*ilvA* Δ*panBilvN*M13 (pECKA*ilvBNC*), cultivated under batch conditions. As AHAS in *C. glutamicum* exhibits a weak affinity to pyruvate - K_M pyruvate=8.3 mM (Leyval et

al. 2003), intracellular pyruvate concentration could be essential in maintaining AHAS activity and L-valine synthesis in cells.

Therefore the effect of pyruvate concentration on AHAS activity and L-valine synthesis rate was further elucidated in short-term flask experiments. Cellular growth limitation by D-pantothenate or oxygen availability were used as methods to increase the intracellular pyruvate level in strains *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) or *C. glutamicum* Δ*ilvA* (pJC1*ilvBNCD*).

3.4.1. Effect of pyruvate concentration on L-valine synthesis under D-pantothenate limited growth conditions

Cellular growth limitation for D-pantothenate was used as a method to increase the intracellular concentration of pyruvate in cells of D-pantothenate auxotrophic strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*). An increase in intracellular pyruvate under D-pantothenate limited conditions could be a result of: (i) restricted CoA formation and therefore restricted pyruvate flow into TCA, (ii) limited cellular growth and consequently limited pyruvate utilisation for biomass formation.

In the first experiment the non-limited (control culture) and D-pantothenate limited cell cultures were recultivated in CGXII minimal medium supplemented with sufficient L-isoleucine concentration. Although there was observed an increase in pyruvate concentration under D-pantothenate limited conditions, no increase in AHAS activity was determined (experimental data not shown). Due to the knowledge that AHAS activity could be inhibited by increased L-isoleucine concentration (Morbach et al. 2000), further D-pantothenate limitation was combined with restricted L-isoleucine availability (0.57 mM instead of 1.14 mM).

Results showed that the biomass formation was reduced under D-pantothenate limitation: while the biomass achieved in a standard medium with sufficient D-pantothenate concentration was 7.33 g l^{-1}, under D-pantothenate limited conditions it was only 5.65 g l-1, consequently 1.3 times lower (Fig. 10A). Intracellular pyruvate concentration was 3.8 times higher in cells grown under conditions of D-pantothenate limitation than in cells grown under D-pantothenate excess (Fig. 10B). Thus, D-pantothenate limitation caused an increase in intracellular pyruvate concentration. Under these D-pantothenate and L-isoleucine limited conditions an increase in intracellular pyruvate concentration resulted in increased AHAS activity and L-valine

synthesis (Fig. 10C, 10E, 10F). The decrease in TCA regulatory enzyme AC activity indicated a decreased pyruvate flow into TCA (Fig. 10D). This could be one of the reasons for the increase in intracellular pyruvate concentration.

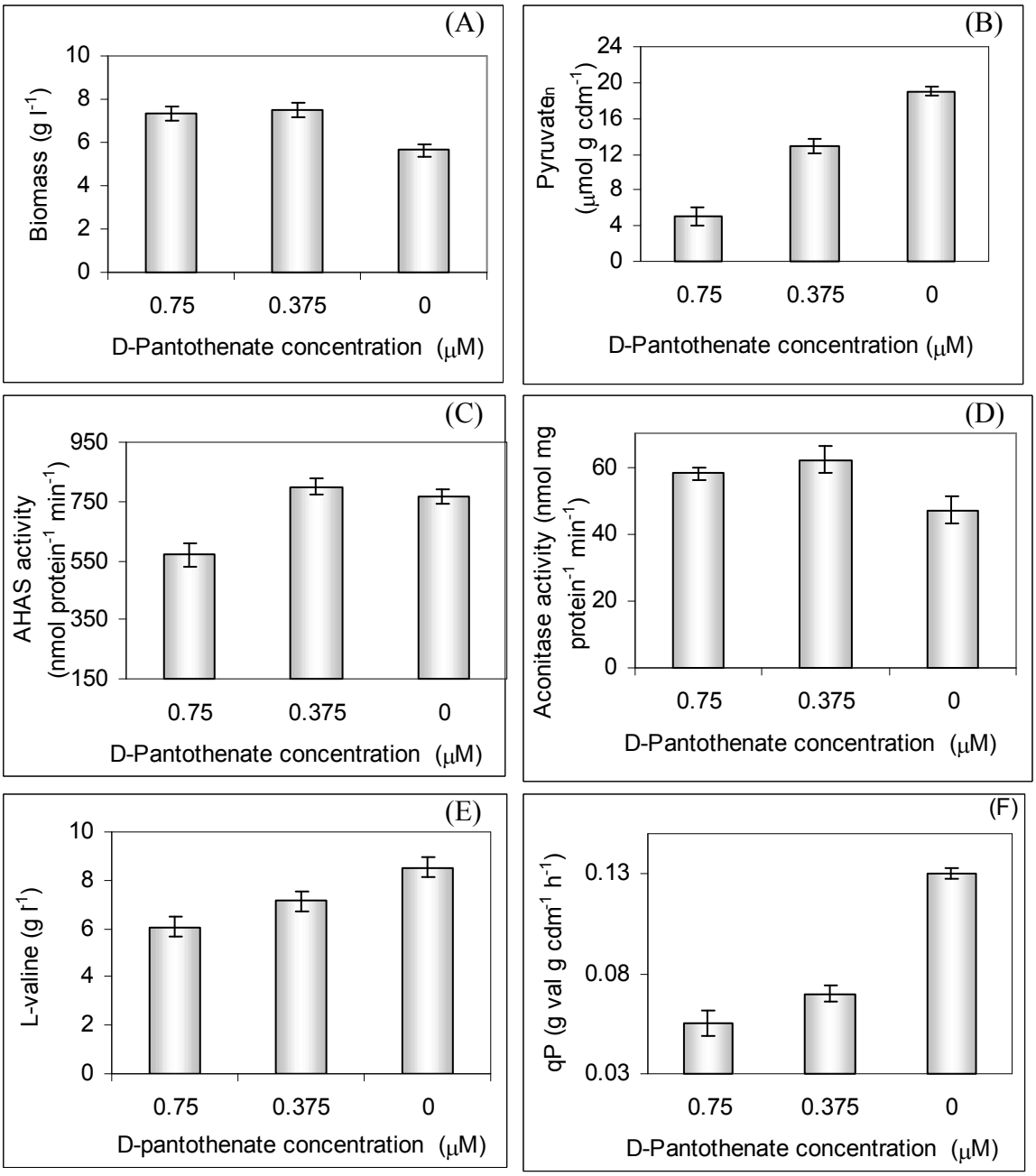

Fig. 10. The effect of D-pantothenate concentration on **(A)** biomass, **(B)** intracellular pyruvate concentration, **(C)** AHAS activity, **(D)** aconitase activity, **(E)** L-valine concentration, **(F)** L-valine synthesis rate of *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNC*). Results of 4h recultivation, L-isoleucine concentration in the medium was 0.75 mM.

Consequently, it was shown that AHAS activity upregulation was more influenced by the decreased L-isoleucine concentration than by the increased intracellular pyruvate concentration alone.

3.4.2. Effect of pyruvate concentration on L-valine synthesis under oxygen limited growth conditions

The cellular growth limitation by oxygen availability was used as another method to decrease the cellular growth and to increase the intracellular pyruvate concentration. Besides, it was shown in chapter 3.2. that a decrease in energy generation in cells was favourable for the increase in L-valine synthesis. To obtain oxygen-limited conditions the exponentially grown cell cultures from baffled shake flasks were transferred to shake flasks without baffles. In this manner the oxygen concentration in the medium was reduced. The cell cultures were recultivated for 4h under applied conditions.

The results showed that biomass formation and the cellular growth rate of strain *C. glutamicum* Δ*ilvA* (pJC1*ilvBNCD*) decreased under conditions of oxygen limitation: biomass concentration achieved under sufficient aeration conditions was 11.78 g l^{-1} while under oxygen limited conditions it was only 7.53 g l^{-1}, consequently 1.6 times lower (Table 3). There was observed a large increase in the intracellular pyruvate concentration under oxygen-limited conditions. However, this increase in the intracellular concentration of pyruvate was not followed by the increase in AHAS activity and L-valine synthesis rate. Therefore it was necessary to elucidate the possible reasons of disparity between a high concentration of intracellular pyruvate and a low AHAS activity. It was presumed that some other enzymes possibly consumed the pyruvate. The decrease in aconitase activity under oxygen limited conditions showed that pyruvate was not required in TCA cycle. Since it is known that under anaerobic conditions pyruvate preferably is converted to lactate (in order to regenerate NAD^+), the activity of lactate dehydrogenase (LDH) was measured. Indeed, there was observed a sharp increase in LDH activity under oxygen-limited conditions in comparison with sufficient aeration conditions. However, it is doubtful whether the decreased AHAS activity could be explained only by the increased LDH activity, because the intracellular pyruvate concentration remained high. The increased NADH dehydrogenase activity under oxygen limited conditions indicated a

high cell demand for NAD^+ regeneration. Thus, the results of these experiments showed that not only an increase in pyruvate concentration but also other intracellular conditions were required for AHAS upregulation.

Table 3. Effect of aeration intensity on biomass, L-valine and intracellular pyruvate concentration and enzyme (LDH, $NADH_2$- dehydrogenase and AC) activity of *C. glutamicum* Δ*ilvA* (pJC1*ilvBNCD*) cells.

Parameters/Aeration intensity	**Sufficient***	**Low****	**Very low*****
x ($g\ l^{-1}$)	11.7±0.55	7.53±0.30	7.16±0.36
L-valine ($g\ l^{-1}$)	1.79±0.09	0.89±0.04	0.93±0.03
$Pyrvate_{in}$ ($\mu mol\ g\ cdm^{-1}$)	0.57±0.03	4.9±0.22	113±0.04
AHAS activity ($nmol\ mg\ protein^{-1}\ min^{-1}$)	466±22	188±9	177±7
q_P ($g\ L\text{-}val\ g\ cdm\ h^{-1}$)	0.051±0.005	0.032±0.005	0.033±0.005
LDH activity ($nmol\ mg\ protein^{-1}\ min^{-1}$)	20±1.2	126±6.5	190±8.7
NADH dehydrogenase activity ($nmol\ mg\ protein^{-1}\ min^{-1}$)	70±3.5	110±4	190±9.2
Aconitase activity ($nmol\ mg\ protein^{-1}\ min^{-1}$)	185±10	118±4.5	47±2

The cell culture was grown exponentially until it reached μ_{max}. Then cells were exposed to different aeration regimes and recultivated for 4h.
*Sufficient aeration: cells were cultured in baffled shake flasks (50 ml medium per 750 ml flask volume)
** Low aeration: cells were cultured in shake flasks without baffles (50 ml medium per 750 ml flask volume)
*** Very low aeration: cells were cultured in shake flasks without baffles and with an increased medium volume per flask (120 ml medium per 750 ml flask volume)

Consequently it was shown that under oxygen limited conditions high intracellular pyruvate concentrations could not provoke an increase in AHAS activity. Therefore severe oxygen limitation did not succeed in increased L-valine synthesis.

Putting the results together it was concluded that an increased pyruvate concentration positively influences L-valine synthesis only under L-isoleucine limited conditions and when sufficient aeration during the cultivation is maintained. The maintenance of NAD^+ regeneration by $NADH_2$-dehydrogenase in cells is also required. Thus, pyruvate probably not always will have the determinative role in AHAS and L-valine synthesis regulation in L-isoleucine auxotrophic *C. glutamicum* recombinants.

3.5. The role of L-valine synthesis enzymes

It was shown above that a correlation between the decrease in cellular growth rate and the increase in AHAS activity and L-valine synthesis rate was observed in all studied strains. But still it was important to elucidate the kinetics of other L-valine synthesis enzymes during batch cultivations. The strain *C. glutamicum* Δ*ilvA* Δ*panB*ilv*N*M13 (pECKA*ilvBNC*) (Elišáková et al. 2005) was used as a model strain in following experiments. The increased resistance of AHAS to inhibition by any of the BCAAs (due to the *ilvN*M13 mutation) is an advantage of this strain in terms of reducing the regulatory effects of varying concentrations of BCAAs on AHAS activity and their physiological consequences.

Strain *C. glutamicum* Δ*ilvA* Δ*panB*ilv*N*M13 (pECKA*ilvBNC*) was grown under batch conditions. Similarly to other strains a decrease in cellular growth rate inversely correlated with an increase in L-valine synthesis (Fig. 11A) and an increase in AHAS activity (Fig. 11B). Compared to AHAS activity, only a slight increase in AHAIR and DHAD activities was observed. The TmB activity remained almost constant during the growth (Fig. 11B). As AHAS specific activity was more than 12-fold higher than AHAIR, DHAD and TmB specific activities and it was strictly upregulated during the growth, this allowed us to conclude that AHAS has the main regulatory role in L-valine synthesis pathway.

However, it was still unclear what exactly caused the AHAS upregulation. As it was shown above in chapter 3.4.1, it was considered that AHAS activity could be more influenced by L-isoleucine concentration than by its substrate pyruvate concentration. Therefore the dynamics of extracellular and intracellular L-isoleucine concentrations during the batch cultivation were determined.

Extracellular and intracellular concentration of L-isoleucine gradually decreased during batch cultivations of *C. glutamicum* Δ*ilvA* Δ*panB*ilv*N*M13 (pECKA*ilvBNC*) (Fig. 11C). It was related with the decrease in cellular growth rate and the increase in activity of AHAS and L-valine synthesis rate (Fig. 11A,B). Hence it was concluded that the possible reason of the growth rate downregulation as well as the AHAS activity upregulation during batch cultivations could be the decreased L-isoleucine concentration.

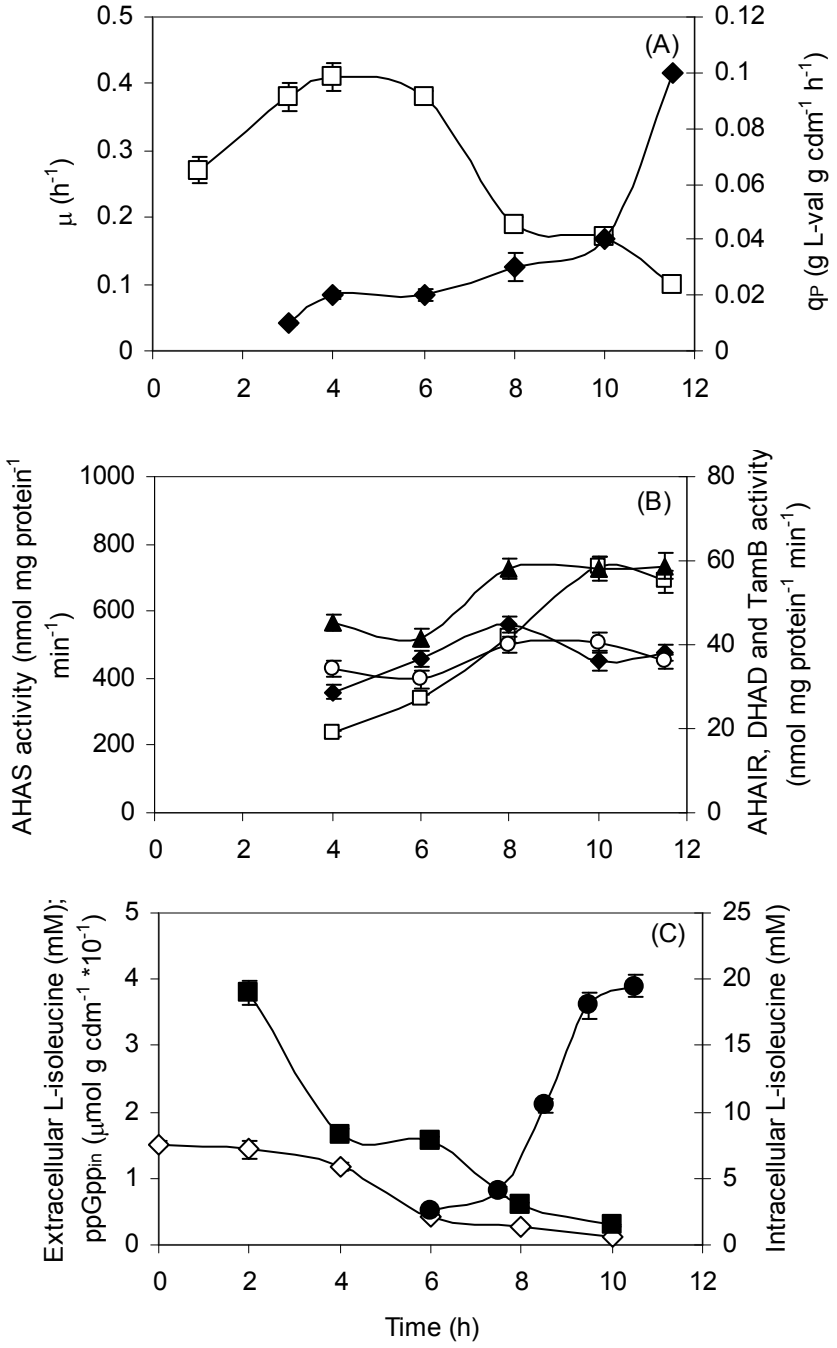

Fig. 11. Batch cultivation of *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*). **(A)** cellular growth rate (□), L-valine synthesis rate (◆); **(B)** activity of AHAS (□), AHAIR (▲), DHAD (◆) and TmB (○); **(C)** L-isoleucine extra (◊) and intracellular (■) concentration, ppGpp intracellular concentration (●).

On the other hand, the stringent response and the alarmone ppGpp under L-isoleucine limited conditions should not be ignored: the stringent response on its own could decrease the cellular growth rate and increase the expression of L-valine synthesis genes. To ascertain whether the stringent response was induced due to L-isoleucine limitation, the intracellular concentration of ppGpp – the regulatory molecule of stringent response – was measured.

The intracellular ppGpp concentration increased sharply between 8-10 h of batch cultivation – a time point when the cellular growth rate already had decreased to 0.1 h^{-1} and a decrease in L-isoleucine intracellular concentration was also observed (Fig. 11C). While the ppGpp concentration was under the detection limit when the cellular growth rate was maximal, the concentration reached 0.39±0.02 μmol g cdm^{-1} after 11 h of cultivation. Thus it was proved that the stringent response was launched. However, this also indicated that two interconnected factors could be involved in the cellular growth rate downregulation and AHAS activity upregulation: (i) L-isoleucine limitation, (ii) L-isoleucine limitation-induced stringent response. The exact role of these parameters on AHAS activity and L-valine synthesis was further investigated.

3.6. Effects of L-isoleucine limitation and stringent response induction on the activity of L-valine forming enzymes and amino acid production

It was shown above that one of the possible reasons causing AHAS activity increase in *C. glutamicum* recombinant cells during batch cultivations was the decrease in L-isoleucine concentration. L-isoleucine concentration in the medium had decreased to 0.1-0.2 mM after 12 hours of cultivation and the cellular growth rate had decreased below 0.1 h^{-1}. But it should be noted that L-isoleucine uptake could be blocked due to its competition with L-valine for uptake by BrnQ, a carrier which transports all branched-chain amino acids into *C. glutamicum* cells (Tauch et al. 1998, Lange et al. 2003). Consequently, the L-isoleucine concentration in the medium could be theoretically sufficient, but the cellular growth rate still decreases, because L-isoleucine uptake is inhibited. For instance, the extracellular L-valine concentration reached about 30 mM at after 12 hours of cultivation (data not shown).

To evaluate the L-isoleucine limitation effect on the cellular growth rate and AHAS activity more properly, short-term experiments in flasks under conditions of full L-isoleucine limitation were carried out. The full L-isoleucine limitation was achieved by recultivating the cell culture in medium lacking L-isoleucine. To compare the full L-isoleucine limitation effect with that of L-isoleucine uptake inhibition, cell cultures where supplemented with various L-valine concentrations.

The most prominent effect of L-isoleucine limitation on the cellular growth rate was observed in cell cultures where 25 mM L-valine was added to the cell culture medium at the beginning of cultivation: the growth rate was lowest in these cells (Table 4). The biomass concentration of these cultures after 8 h of cultivation was also

about ½ half of that achieved in the control cultures version (data not shown). The higher AHAS activities in strain *C. glutamicum* Δ*ilvA*Δ*panB ilvN*M13 (pECKA *ilvBNC*) points to a positive effect of the introduced feedback resistance (*ilvN*M13) on enzyme activity.

Table 4. The effect of L-isoleucine concentration or L-isoleucine uptake limitation on the cellular growth rate and AHAS activity in cells of different *C. glutamicum* recombinant strains.*

	μ ($g\ l^{-1}$)	**AHAS activity** (nmol mg $protein^{-1}$ min^{-1})
(A) *C. glutamicum* Δ*ilvA* (pJC1 *ilvBNCD*)		
Control (+ 1.14 mM L-Ile)	0.45±0.05	104±5
I Analyses (without L-ile)	0.26±0.02	333±12
II Analyses (+ 1.14 mM L-Ile + 25 mM val)	0.16±0.02	530±20
III Analyses (+ 1.14 mM L-Ile + 100 mM val)	0.24±0.02	251±12
(B) *C. glutamicum* Δ*ilvA*Δ*panB ilvN*M13 (pECKA *ilvBNC*)		
Control (+ 1.14 mM L-Ile)	0.32±0.03	185±9
I Analyses (without L-ile)	0.24±0.02	475±21
II Analyses (+ 1.14 mM L-Ile + 25 mM val)	0.17±0.02	674±37
III Analyses (+ 1.14 mM L-Ile + 100 mM val)	0.20±0.03	591±20

* Experimental conditions: cell cultures were grown exponentially until μ reached μ_{max}. Then cells were recultivated in CGXII medium:

- Control: with 1.14 mM L-isoleucine;
- I Analyses: without L-isoleucine;
- II Analyses: with 1.14 mM L-isoleucine and 25 mM L-valine supplemented at the beginning of experiment;
- III Analyses: with 1.14 mM L-isoleucine and 100 mM L-valine supplemented at the beginning of recultivation.

Yet an increase in AHAS activity due to L-isoleucine limitation was observed in every case, no matter how the starvation of L-isoleucine was achieved. The highest AHAS activity was achieved under conditions when 25 mM L-valine was added to the medium at the beginning of cultivation. Thus it was shown that a restricted L-isoleucine availability already from the early phases of cultivation possibly enables a full derepression of *ilvBN* gene transcription (Morbach et al. 2000). Consequently, the obtained results showed that L-isoleucine limitation had a direct positive effect on AHAS activity.

The question arose whether the increase in AHAS activity was caused by the L-isoleucine limitation itself or the L-isoleucine limitation-induced stringent response. To elucidate the possible effect of the stringent response on growth, L-valine enzyme activity and L-valine synthesis *rel* proficient (*C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13

pECKA*ilvBNC*) and *rel* deficient (*C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 Δ*rel* pECKA*ilvBNC*) strains were used in further study. Due to the *rel* deletion ppGpp was not synthesized in *rel*$^-$ cells.

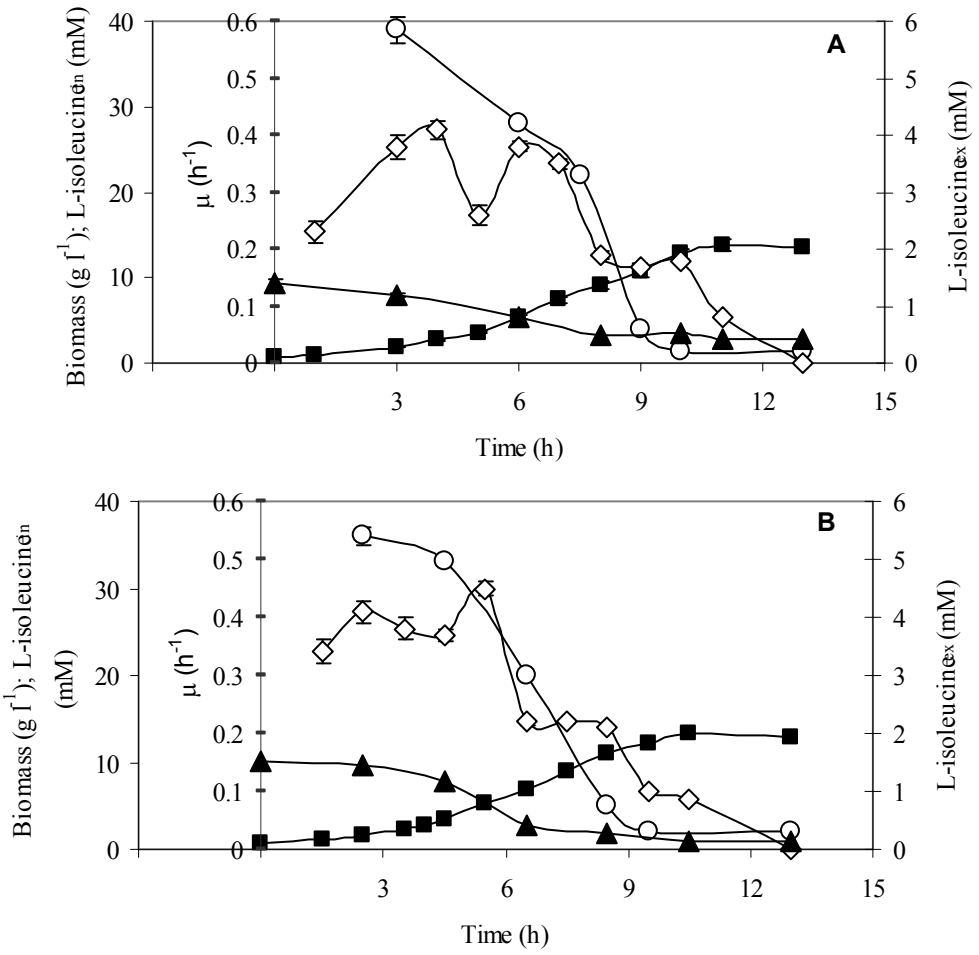

Fig. 12. Fed-batch cultivations of *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) (A) and its *rel*$^-$ derivative (B). **(A)** and **(B)**: biomass (■), cellular growth rate (◊), extracellular (▲) and intracellular (○) L-isoleucine;

The exponential growth under L-isoleucine excess, as well as the reduction of the growth rate as a consequence of L-isoleucine depletion in the medium and inside the cells (after 6 hours of cultivation, Fig. 12A, B), were comparable for the *rel*$^+$ and *rel*$^-$ strains (Fig. 12 A,B). The stringent response therefore did not induce an apparent effect on the cellular growth rate. Note that a similar, though temporary drop in the growth rate of both strains after 4 hours of cultivation also occurred. This temporary drop in the growth rate was quite unexpected, as it was not related to L-isoleucine depletion. It coincided with a rather sharp increase in intracellular L-valine in *rel*$^+$ and

rel$^-$ cells although its extracellular accumulation was low. The reason for the atypical growth kinetics of *rel*$^+$ and *rel*$^-$ recombinants of *C. glutamicum* during the fed-batch cultivations is not yet clear.

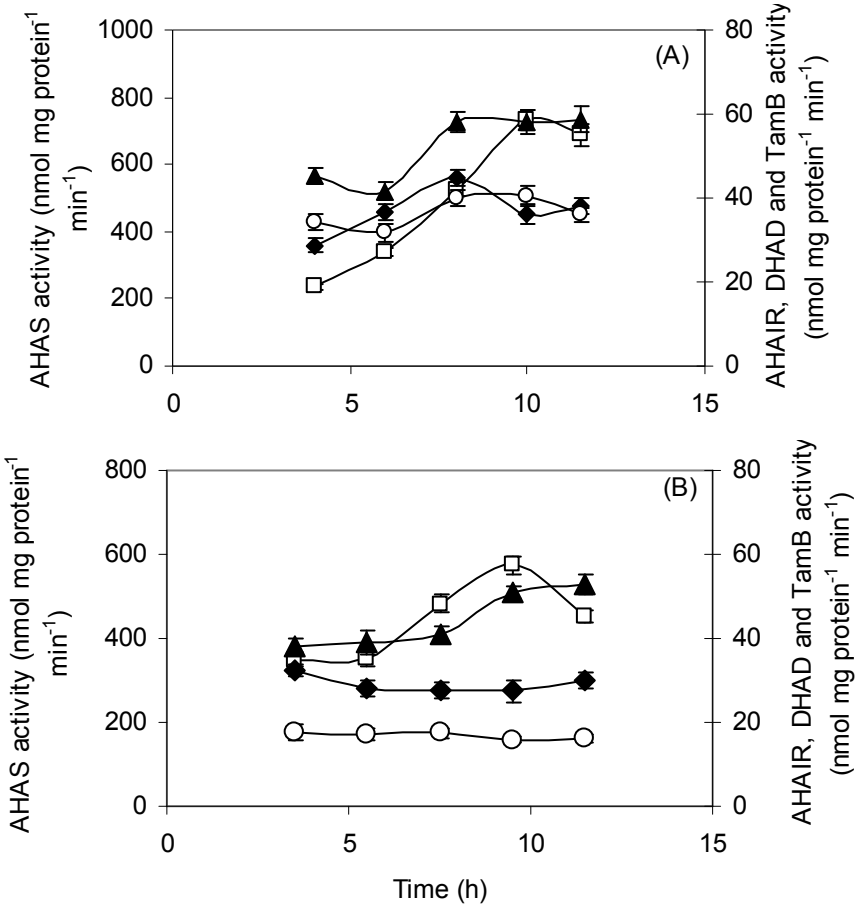

Fig. 13. Fed-batch cultivations of *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) (A) and its *rel*$^-$ derivative (B). **(A)** and **(B)**: activity of AHAS (□), AHAIR (▲), DHAD (♦) and TmB (○)

There was also observed a similar increase in AHAS activity in both strains due to the decrease in L-isoleucine limitation (Fig. 13A,B). Consequently it might be considered that the increase in AHAS activity was mostly due to the decrease in L-isoleucine concentration. AHAIR – the second enzyme that could be directly positively influenced by L-isoleucine limitation – showed a slight increase in *rel*$^+$ and *rel*$^-$ strains after the cellular growth rate downregulation. DHAD showed a slight increase in *rel*$^+$ but remained quite stable in *rel*$^-$ strain. TmB activity remained stable in both strains during the cultivation (Fig. 13 A,B). Thus, the results showed that AHAS and AHAIR were upregulated due to L-isoleucine limitation and this effect

was similar in *rel*$^+$ and *rel*$^-$ cells, and no effect of the stringent response induction on these enzymes was observed. Yet remarkable was a lower DHAD and TmB activity in *rel*$^-$ strain as compared to the enzyme activity in the *rel*$^+$ strain.

The stringent response had an effect on L-valine synthesis rate: whereas the rate of L-valine synthesis remained high in the *rel*$^+$ strain, it significantly decreased in the *rel*$^-$ strain during the stationary growth phase (Fig. 14A, B). The L-valine synthesis rate of *rel*$^+$ strain was 1.5-fold higher (q_P=0.10 g L-val g cdm^{-1} h^{-1}) than that achieved by *rel*$^-$ strain (q_P=0.065 g L-val g cdm^{-1} h^{-1}) (Fig. 14A, B). The achieved L-valine concentration after 12 h of cultivation was 1.6 times higher in the stringent strain than that in the relaxed strain (Fig. 14A, B).

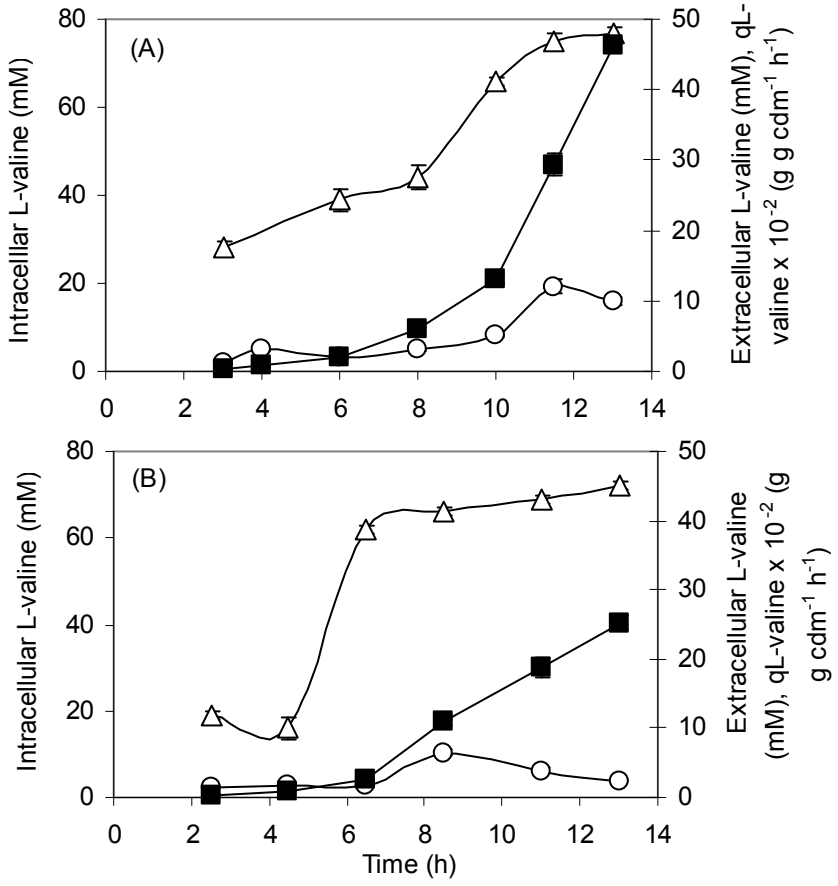

Fig. 14. Fed-batch cultivations of *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) (A) and its *rel*$^-$ derivative (B). Intracellular (Δ) and extracellular (■) L-valine and rate of L-valine synthesis (○).

It should be also mentioned that there was observed a slight increase in the synthesis of side amino acids in *rel*$^-$ strain (Fig. 15). No remarkable differences in the dynamics of intracellular pyruvate concentration and G-6-PDH activity were observed between *rel*$^+$ and *rel*$^-$ strains during fed-batch cultivations (data not shown).

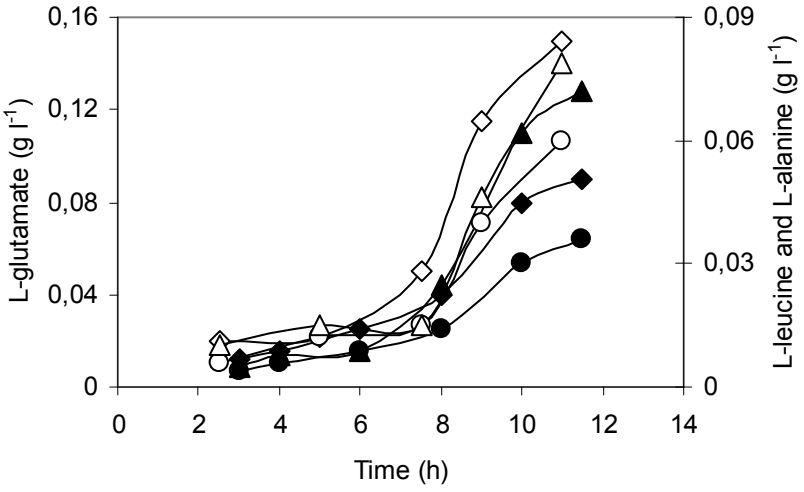

Fig. 15. Amino acid synthesis in *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) (filled symbols) and its *rel*$^-$ derivative (empty symbols) during fed-batch cultivations. L-glutamate (♦, ◊), L-leucine(▲, Δ), and L-alanine (●, o).

To document the observed effects of L-isoleucine limitation and the stringent response induction on the activity of the enzymes of the L-valine synthesis pathway in the tested *C. glutamicum* strains, short-term recultivations of the exponentially growing *rel*$^+$ and *rel*$^-$ cells under conditions of L-isoleucine excess (medium with 1.52 mM L-isoleucine) and deficiency (L-isoleucine-free medium supplemented by 10 mM L-valine) were carried out. A complete depletion of intracellular L-isoleucine was achieved after a 1-h recultivation of the cells in the L-isoleucine-free medium**.**

The growth was significantly reduced due to L-isoleucine limitation (Table 5): the biomass concentration in the cell culture subjected to L-isoleucine starvation for 3 h was 1.4 times lower in comparison with the concentration achieved in a standard medium in both *rel*$^+$ and *rel*$^-$ strains. These data confirmed the results obtained in batch cultivations indicating that the cellular growth is regulated by L-isoleucine concentration and this regulation might be *rel*-independent. The L-isoleucine limitation resulted in a significant increase in AHAS and AHAIR activity in both *rel*$^+$

and *rel*$^-$cells. In *rel*$^+$ cells it also resulted in a slight increase in DHAD activity, but in the *rel*$^-$ cells a significant decrease in this enzyme activity was apparent.

Table 5 Effect of L-isoleucine availability on biomass formation, cellular respiration and enzyme activity in *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*)[a,b] and its *rel*$^-$ derivative

	Strain			
	***rel*$^+$**		***rel*$^-$**	
	L-isoleucine availability			
Parameters	**Excess**	**Limitation**	**Excess**	**Limitation**
X (g l^{-1})	6.87 ± 0.55	4.80 ± 0.32	6.45 ± 0.45	4.63 ± 0.40
AHAS activity (nmol mg protein min^{-1})	451 ± 24	556 ± 32	364 ± 16	437 ± 18
AHAIR activity (nmol mg protein min^{-1})	68 ± 5	88 ± 7	43 ± 4	56 ± 3
DHAD activity (nmol mg protein min^{-1})	36 ± 4	44 ± 4	13 ± 2	7 ± 1
TmB activity (nmol mg protein min^{-1})	32 ± 2	16 ± 3	17 ± 2	16 ± 2

[a] Results of 4-hour recultivation under L-isoleucine excess or limitation (L-isoleucine-free medium supplemented with 10 mM L-valine) conditions
[b] Data represent average values ± SD for three independent cultures

Variations in cellular respiration during cultivations of *rel*$^+$ and *rel*$^-$ strains were further investigated in order to reveal the possible reasons for low L-valine synthesis in slow-growing stringent negative cells. It was shown that an increased enzyme activity and L-valine synthesis in *rel*$^+$ cells related to an increased ATP synthesis-coupled and a decreased ATP synthesis-uncoupled respiration. The $Q_{O2\ CN\text{-}sensitive}$ of *rel*$^+$ cells subjected to L-isoleucine limitation was approximately 2-fold higher but $Q_{O2\ CN\text{-}resistant}$ was approximately 2-fold lower compared to that of *rel*$^-$ cells (Fig. 16).

Increased cyanide-resistant respiration in *C. glutamicum* was previously reported to result from reactions in which molecular oxygen chemically oxidizes redox enzymes, forming a superoxide (Shvinka et al. 1979). Therefore, the aforementioned differences in the respiration of *rel*$^-$ as compared to *rel*$^+$ cells could be an indication of an increased oxidative stress in stringent-negative cells exposed to L-isoleucine limitation.

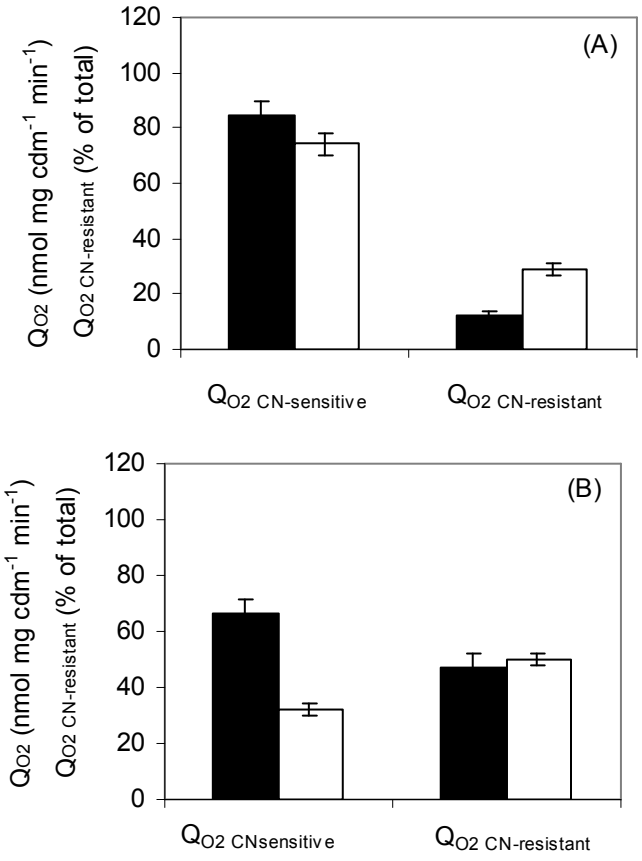

Fig. 16. Effect of L-isoleucine limitation on ATP-synthesis coupled and uncoupled respiration ($Q_{O2\ CN\text{-}sensitive}$ and $Q_{O2\ CN\text{-}resistant}$) in cells of (A) *rel*$^{+}$ and (B) *rel*$^{-}$ strains of *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*). Cells were grown exponentially under L-isoleucine abundant conditions, then recultured for 4 h in L-isoleucine abundant (*filled bars*) or L-isoleucine free medium supplemented with 10 mM L-valine (*empty bars*).

Consequently, our results showed that the positive effect of the decrease in the cellular growth rate on L-valine synthesis rate could be explained by the positive effect of L-isoleucine limitation on L-valine synthesis enzyme AHAS upregulation, and the stringent response is not involved in the enzyme upregulation. The L-isoleucine limitation might be the biochemical basis for the increase in AHAS activity, and the intracellular pyruvate concentration would have a secondary role in the enzyme regulation. The presence of the *rel* gene is beneficial for maintaining L-valine synthesis during the stationary growth phase.

3.7. Development of the technology to enhance L-valine overproduction during fed-batch cultivations

3.7.1. Maintenance of the cellular growth during the stationary phase

A low L-valine production, in spite of a high AHAS activity and internal pyruvate concentration, was observed during fed-batch cultivations of strains *C. glutamicum* Δ*ilvA* (pJC1*ilvBNCD*) and *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) when the cells entered the stationary growth phase and μ dropped below 0.05 h^{-1} (chapter 3.2). Therefore, a method to improve the growth and L-valine production by the stationary growth phase cells was developed.

As it was described above, the growth rate of cells of L-isoleucine auxotrophs during fed-batch cultivations mainly depend on L-isoleucine concentration. In further experiments addition of small doses of L-isoleucine to a stationary growth phase cell culture in a bioreactor was used to maintain the cellular growth rate above 0.05 h^{-1} and to elucidate its effect on L-valine production. A slow-growing cell culture of strain *C. glutamicum* Δ*ilvA* (pJC1*ilvBNCD*) after 12 hours of its fed-batch cultivation in a bioreactor was supplemented with 0.03 g l^{-1} L-isoleucine. Changes in the rates of cellular growth and L-valine synthesis, the amino acid precursor concentrations and the activity of enzymes of central metabolism and L-valine synthesis were measured after 4 and 6 hours of L-isoleucine supplementation.

It was shown that L-isoleucine supplementation to the cell culture after 12 h of cultivation in a bioreactor helped to maintain the growth rate of about 0.05 h^{-1} (Table 6) whereas the cellular growth rate decreased to 0.001 h^{-1} after 18 h of cultivation when no L-isoleucine was supplemented to the cell culture. Further, the effect of L-isoleucine supplementation on L-valine synthesis was investigated. L-valine synthesis rate was 2-fold higher in the cell culture supplemented with L-isoleucine (q_P was 0.074 and 0.035 g val g cdm^{-1} h^{-1} respectively). However, there was observed no influence of L-isoleucine supplementation on the intracellular pyruvate concentration and AHAS activity in cells. At the same time a noteworthy effect of L-isoleucine supplementation was observed on NADPH concentration and G-6-PDH activity. The activity of G-6-PDH was 2.5-fold higher and NADPH concentration was 3.5-fold higher in the cells supplemented with L-isoleucine.

Table 6. Variations in physiology and intracellular biochemistry of slow-growing *C. glutamicum* ATCC 13032 Δ*ilvA*Δ*panBC* pJC1*ilvBNCD* cell culture in response to 0.03 g l^{-1} L-isoleucine supplementation in the bioreactor.

Cultivation time (h)	μ (h^{-1})	q_P (g L-val g $cdm^{-1}h^{-1}$)	Intracellular concentration (μmol g cdm^{-1})		Enzyme activity (nmol mg $protein^{-1}$ min^{-1})		
			Pyruvate	NADPH	AC	G-6-PDH	AHAS
Stationary phase cell culture *							
12	0.050 ± 0.0020	0.081 ± 0.0030	5.9 ± 0.17	0.60 ± 0.020	16.0 ± 0.87	28.0 ± 0.58	873 ± 34
A Culture without L-isoleucine supplementation							
16	0.020±0.001	-	4.8±0.2	0.35±0.01	11.2±0.4	11.6±0.4	880±32
18	0.001±0.0001	0.035±0.0017	3.7±0.18	0.09±0.005	6.0±0.26	9.8±0.46	860±22
B Culture with 0.03 g l^{-1} L-isoleucine supplementation							
16	0.050 ± 0.0017	-	5.1 ± 0.20	0.55 ± 0.017	15.0 ± 0.72	26.0 ± 1.32	840 ± 33
18	0.040 ± 0.0020	0.074 ± 0.0033	4.4 ± 0.17	0.32 ± 0.017	12.0 ± 0.30	26.0 ± 0.52	890 ± 41

*Stationary phase cell culture has been grown under batch cultivation conditions for 12 h.

The results described in Table 6 showed that maintenance of the cellular growth rate at a level 0.05 h^{-1} could be important to maintain sufficient G-6-PDH activity and NADPH concentration in cells, thus eliminating this nucleotide limit for L-valine synthesis, and to achieve a satisfactory L-valine synthesis rate by the stationary growth phase cell culture.

3.7.2. Optimisation of aeration intensity during cultivations to increase L-valine synthesis

It was shown previously that a decrease in energy generation in *C. glutamicum* recombinant strains during batch cultivations was favourable for an increase in L-valine synthesis. Besides, it was reported previously that L-valine synthesis can be improved under oxygen limited conditions (Akahashi et al. 1977). However, in the experiment where the cells were cultured under oxygen limited conditions (and thus the energy generation in cells was reduced) no increase in L-valine synthesis was observed although there was a drastic increase in the intracellular pyruvate concentration. At the same time a sharp increase in LDH activity was observed indicating that NAD^+ regeneration in cells was disturbed. It allowed concluding that changes in aeration should be subtler.

Therefore in further experiments changes in the aeration intensity by changing pO_2 profiles during fed-batch cultivations of *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) were performed. The effect of the changes in cellular energy state on the growth and L-valine synthesis was elucidated.

When pO_2 was maintained in the range 35±3 % from saturation during the cultivation, a high maximal cellular growth rate (μ=0.36 h^{-1}) (data not shown) and high biomass concentration were achieved (x=11.5 g l^{-1}) (Fig. 17A). Cell respiration activity remained high during the exponential growth (Fig. 17B). L-valine synthesis rate was rather low: $q_{P\ max}$ was 0.056 g L-val g cdm^{-1} h^{-1} (Fig. 17A). An increase in L-valine synthesis rate followed the decrease in cell respiration activity and the increase in CN-resistant oxygen consumption, uncoupled to ATP generation in cells (Fig 17B). Thus, the maintenance of pO_2 35±3 % from saturation resulted in a high cell respiration activity, high biomass concentration but low L-valine synthesis.

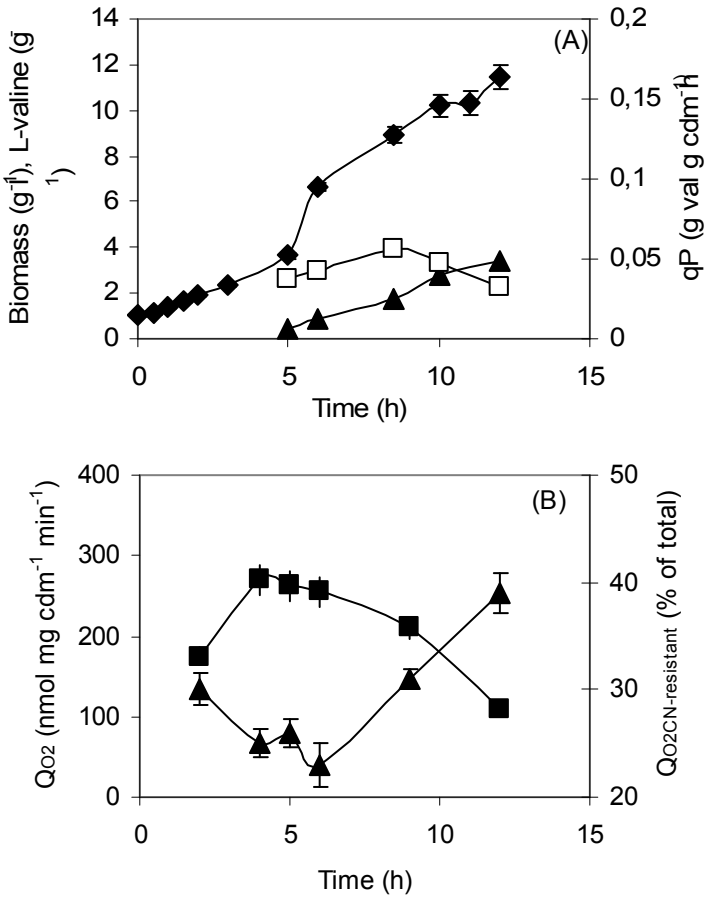

Fig. 17. Changes in **(A)** biomass (♦), and L-valine (▲) concentration, and L-valine synthesis rate (□); **(B)** cellular "summary" (●) and CN-resistant respiration activity during the fed-batch cultivation of Δ*ilvA*Δ*panBC* pJC1*ilvBNCD*. pO_2 35±3 % from saturation during the cultivation was maintained.

A further attempt to decrease the cell energy generation was made by maintaining pO_2 15±3 % from saturation during the cultivation. It was shown that the cell respiration activity was lower but CN-resistant oxygen consumption increased sharply and reached about 57% of total (Fig. 18B). It resulted in a high L-valine concentration and a high L-valine synthesis rate (q_{Pmax} was 0.17 g L-val g cdm^{-1} h^{-1}) (Fig 18A).

Unfortunately the achieved biomass concentration was only 7 g l^{-1} under aeration conditions of pO_2 15±3 % (Fig. 18A). It was presumed that the induction of high L-valine synthesis activity in the early stages of bacterial growth and the following increased L-valine concentrations in the medium (above 40 mM) could inhibit the L-isoleucine uptake in cell (Lange et al. 2003) and consequently inhibit the cellular growth rate and biomass formation.

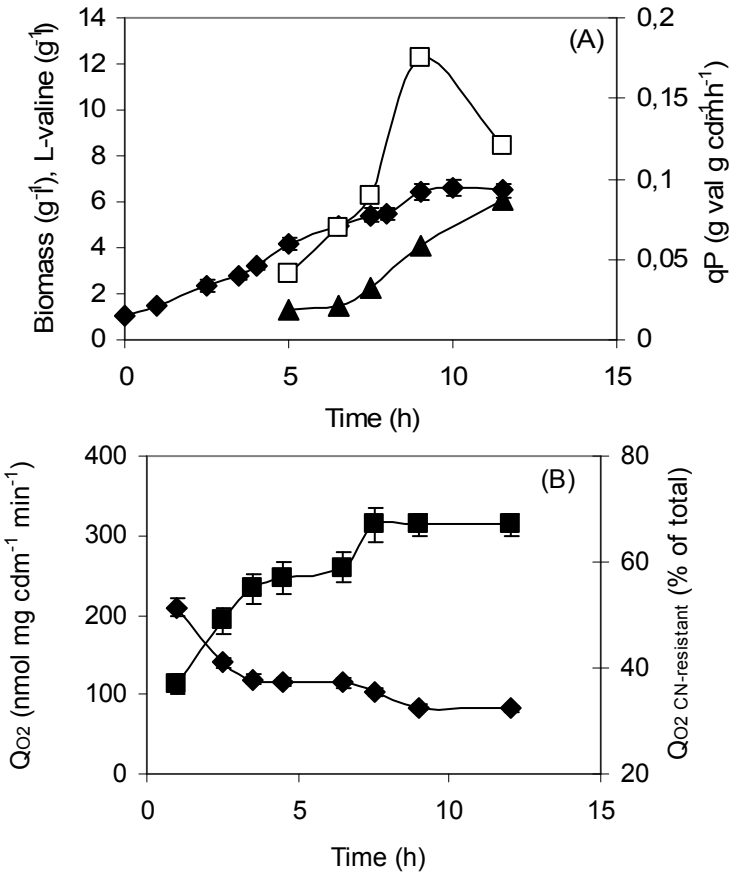

Fig. 18. Changes in **(A)** biomass (♦), and L-valine (▲) concentration, and L-valine synthesis rate (□); **(B)** cellular "total" (●) and CN-resistant respiration activity during the fed-batch cultivation of Δ*ilvA*Δ*panBC* pJC1*ilvBNCD*. pO_2 15±3 % from saturation during the cultivation was maintained.

Table 7. The effect of different regimes of aeration intensity on the growth, AHAS, L-valine synthesis and respiration activity during fed-batch cultivations of Δ*ilvA*Δ*panBC* pJC1*ilvBNCD*.

Parameters	**pO_2=35 ± 3 %**	**pO_2=15 ±3 %**	**pO_2=25 ± 3 % (0-7h) 17±3 % (7-15h)**
X_{12h} ($g\ l^{-1}$)	11.5±0.6	7.0±0.35	8.0±0.3
P_{12h} ($g\ l^{-1}$)	2.8±0.12	5.5±0.3	6.0±0.3
q_{P8h} (g val g cdm-1 h-1)	0.046±0.01	0.175±0.01	0.185±0.03
$q_{Paverage}$ (g val g cdm-1 h-1)	0.046±0.01	0.130±0.005	0.148±0.004
$Q_{O2\ 4h}$	270±19	116±6	197±10
Q_{O2} CN res$_{4h}$	25±2	57±3	30±2
AHAS activity (nmol mg $protein^{-1}$ min^{-1})	317±15	718±20	883±17
$Pyruvate_{in}$ (μmol g cdm^{-1})	4.2±0.3	8.9±0.5	11.5±0.5

Therefore a strategy to maximize L-valine synthesis was developed: pO_2 was adjusted at 25±3 % until half of X_{max} was reached, than pO_2 was decreased to 17±3 %. Using this method x=8 g l^{-1} per 1.14 mM L-isoleucine and q_P=0.15±0.03 g val g cdm^{-1} h^{-1} during bacterial growth in bioreactor were maintained (Table 7).

Thus it was shown that a slight decrease in the cellular energy generation (a decrease in TCA activity and cellular respiration activity) enhances L-valine synthesis, but at the same time an induction of L-valine synthesis at an early growth phase is not favourable for the final product outcome.

4. DISCUSSION

A considerable progress has been achieved in the strain construction of a wild type *C. glutamicum* ATCC 13032 to increase L-valine overproduction by redirecting precursor flow and reducing the feedback inhibition of L-valine synthesis enzymes by branched-chain amino acids (Sahm et al. 1999, Radmacher et al. 2002, Elišáková et al. 2005). However, L-valine synthesis by the constructed strains varies significantly depending on the physiological state of cells during the cultivations. Therefore the aim of the recent work was to characterize the optimum cellular physiology and biochemical basis for enhancing L-valine overproduction by L-isoleucine auxotrophic *C. glutamicum*. Various recombinant strains were used in this study to investigate the essential parameters increasing L-valine synthesis by objective considerations.

First of all, various *C. glutamicum* recombinant strains were evaluated for L-valine synthesis capability. It was shown here that additional *panBC* deletion had more preferences for L-valine synthesis than single *ilvA* deletion. L-valine synthesis rate (q_P) and L-valine yield from substrate ($Y_{P/S}$) achieved by strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) were 0.13 g L-val g $cdm^{-1}h^{-1}$ and 0.45 g L-val g $glucose^{-1}$, respectively, but q_P and $Y_{P/S}$ achieved by an analogous strain, only without *panBC* deletion, were 0.087 g L-val g $cdm^{-1}h^{-1}$ and 0.31 g L-val g $glucose^{-1}$, respectively (Ruklisha et al. 2007). The positive effect of *panBC* deletion is explained by (i) reduced pyruvate flow in TCA (due to the decrease in the pyruvate dehydrogenase acivity) and (ii) increased availability of α-ketoisovalerate.

It was shown that the increased intracellular content of L-valine synthesis enzymes, achieved by *ilvBN*, *ilvC* and *ilvD* overexpresion on plasmid, plays a very important role in the metabolite flux redirection towards L-valine. Although strains without introduced plasmids are preferred in production processes (because these plasmids carry antibiotic resistance genes), unfortunately these strains showed a lower capability of L-valine synthesis. If q_P and $Y_{P/S}$ achieved by strain *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 with overexpressed *ilvBNC* genes were 0.105 g L-val g $cdm^{-1}h^{-1}$ and 0.26 g L-val g $glucose^{-1}$, then q_P and $Y_{P/S}$ achieved by the respective strain without gene overexpression were 0.053 g L-val g $cdm^{-1}h^{-1}$ and 0.078 g L-val g $glucose^{-1}$,

respectively (experimental data not shown). Strains without *ilvBNC* or *ilvBNCD* overexpression showed increased synthesis of side amino acids as well: 1.04 g l^{-1} L-alanine and 0.08 g l^{-1} L-leucine were excreted in the medium in comparison with 0.16 g l^{-1} L-alanine and 0.04 g l^{-1} L-leucine for the strain with *ilvBNC* overexpression (experimental data not shown). It was also shown that the basis vector itself could influence L-valine synthesis capability. Although strains Δ*ilvA* pECM3*ilvBNCD* and Δ*ilvA* pJC1*ilvBNCD* both had *ilvA* gene deleted and *ilvBNCD* overexpressed, the strain bearing plasmid pECM3*ilvBNCD* showed a lower L-valine synthesis rate.

Various reports on L-lysine synthesis reveal that the productivity of L-lysine depends on the cellular growth rate (Kiss and Stephanopoulos 1991, Ruklisha et al. 1993, Lee et al. 1995, Zhao and Lin 2002). Analysing the relationship between the cellular growth and L-valine synthesis, it was shown that an increase in L-valine synthesis was reached when cellular growth decreased below maximum in all studied strains. All L-valine synthesis characterizing parameters – AHAS activity, L-valine intracellular concentration, L-valine synthesis rate, and L-valine experimental yield increased and reached maximal values after cellular growth rate downregulation. However, a low L-valine synthesis activity (q_P=0.02 g L-val g $cdm^{-1}h^{-1}$), despite of a high AHAS activity, was observed when cells entered the stationary phase and cellular growth rate dropped below 0.05 h^{-1} in all studied strains.

In order to explain possible extracellular and intracellular conditions enhancing the increase in L-valine synthesis rate after the cellular growth rate downregulation in L-valine overproducing *C. glutamicum* strains, cellular physiology and intracellular biochemistry during batch and fed-batch cultivations were analysed.

The decrease in the cellular growth rate during batch cultivations correlated with a decrease in energy generation in cells (characterized by cellular total reducing activity, CN-sensitive respiration activity, coupled to ATP synthesis, and cellular energy charge value). A correlation between the decrease in the activity of aconitase, previously established as a regulatory enzyme of the TCA cycle in *C. glutamicum* (Ruklisha et al. 1987), and a decrease in the cellular growth rate was observed as well. Thus it can be concluded that the decrease in TCA cycle functioning and energy generation might be advantageous for an increase in L-valine synthesis in *C. glutamicum* cells. The decrease in aconitase activity and subseqyently TCA acivity

could decrease the pyruvate demand in TCA cycle and consequently increase the pyruvate availability for L-valine synthesis. The decrease in energy generation by oxidative phosphorylation in cells might be advantageous due to an increase in the rate of glucose uptake by cells (Ruklisha et al. 1981). It was also previously reported that the decrease in TRA and energy generation in cells was related with the increase in L-lysine synthesis in *C. glutamicum* (Ruklisha and Paegle 2001). The decrease in energy generation due to reduced H^+-ATPase activity was used as a method to increase L-valine, L-glutamate, L-alanine and pyruvate production in *E. coli* and *C. glutamicum* (Yokota et al. 1994, Aoki et al. 2005, Wada et al. 2007, Wada et al. 2008).

To elucidate whether the increase in L-valine synthesis rate after the cellular growth rate downregulation below maximum was connected with the increase in the intracellular precursor availability and with a switch in central metabolism pathways, the intracellular concentrations of L-valine precursors and the activity of key enzymes of central metabolism and L-valine synthesis pathways were determined. The increase in AHAS activity was related to the increase in the intracellular pyruvate concentration in strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*). The increase in L-valine synthesis rate correlated with the increase in G-6-PDH activity in this strain as well.

The increase in intracellular pyruvate concentration might be considered as basis for an increase in AHAS activity, especially considering the data that AHAS in *C. glutamicum* has a relatively weak affinity for pyruvate (K_m = 8.3 mM) (Leyval et al. 2003) compared to those of enzymes competing with AHAS for pyruvate, e.g. that of pyruvate dehydrogenase (K_m=1.7 mM) (Schreiner et al. 2005) or that of pyruvate carboxylase (K_m=1.3 mM) (Peters-Wendisch et al. 1997). The strong effect of the increase in intracellular pyruvate level on AHAS activity in strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*) has been recently demonstrated during glucose stimulus-response experiments: a 3-fold increase in the intracellular pyruvate concentration resulted in a 25-fold increase in AHAS rate and in a significant increase in L-valine production by *C. glutamicum* (Magnus et al. 2006). The significant role of pyruvate concentration was also demonstrated by Blombach et al. (2007[b], 2008): very high L-valine productivity was achieved due to inactivation of pyruvate consuming enzymes pyruvate dehydrogenase complex and pyruvate:quinone oxidoreductase. The

positive effect of the increased pyruvate concentration, resulting from the cellular growth rate downregulation, on L-lysine synthesis by *C. glutamicum* and *C. lactofermentum* strains has also been reported previously (An et al. 1999, Eggeling and Sahm 1999, Ruklisha and Paegle 2001).

The possible positive effect of the increased pyruvate concentration on AHAS activity and L-valine synthesis rate in this investigation was studied using two methods to increase pyruvate concentration in cells of strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*): (i) D-pantothenate limitation (ii) oxygen limitation. Although an increase in intracellular pyruvate concentration was observed in both cases, it was not necessarily resulting in an increased activity of AHAS and L-valine synthesis rate. It was necessary to combine the D-pantothenate limitation with L-isoleucine limitation to achieve an increase in AHAS activity. Thus, not only the pyruvate concentration alone was important for AHAS upregulation in L-isoleucine auxotrophic *C. glutamicum* strain under D-pantothenate limited conditions. Opposite to this finding are the results of a recent study by Blombach et al. (2007[b], 2008) where L-valine production was dramatically increased due to the elevated pyruvate concentration although L-valine overproducing *C. glutamicum* strains were not auxotrophic for L-isoleucine. Thus we may conclude that role of the pyruvate in L-valine overproduction partly will depend on the genetic background of the production strain.

Under oxygen limited conditions an even more dramatic increase in the pyruvate concentration (about 20-fold) was achieved, but again no increase in AHAS activity was observed. Since a high increase in LDH activity was observed in cells subjected to oxygen limitation, it was suggested that pyruvate could be partly consumed by LDH to synthesize L-lactate and thus to maintain NAD^+ regeneration (during limited oxygen availability NAD^+ regeneration in cells could be disturbed). An increased expression of *ldh* and a decreased expression of *acn* were likewise demonstrated under oxygen deprivation conditions during organic acid production in *C. glutamicum* (Inui et al. 2007). However, the intracellular pyruvate concentration in cells was quite high, and it was an indication that low AHAS activity is not related solely to the pyruvate use for L-lactate synthesis. Hence we may conclude that other mechanisms influencing AHAS and L-valine synthesis regulation under oxygen limited conditions might be involved and could be an aim for the further study.

Opposite to strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*), increased AHAS activity and overproduction of L-valine (and other pyruvate-derived amino acids) in *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) under batch cultivation conditions was related to a decrease in the intracellular pyruvate level. It was suggested that pyruvate was utilized efficiently for amino acid synthesis, as the L-valine synthesis rate was increasing. Thus, the upregulation of pyruvate derived amino acid synthesis in the growth limited cells of this strain was not found to be due to increased intracellular pyruvate concentration, but due to an increased rate of precursor utilization for amino acid synthesis.

Thus putting together the data from the batch cultivations of different strains and short-term experiments it was demonstrated that in L-isoleucine auxotrophic *C. glutamicum* strains the increased pyruvate concentration would enhance AHAS activity and L-valine synthesis rate under conditions of L-isoleucine limitation and when sufficient aeration intensity during cultivation is maintained. It should be noted that a coordinated increase in precursor concentration and availability of L-valine synthesis enzymes is very important. In this study it was demonstrated using strains with or without L-valine synthesis genes overexpressed on multicopy plasmid: less L-valine and high by-product synthesis was achieved in plasmid-less strains (experimental data not shown). Bartek et al. (2008) demonstrated that 35-fold increase in pyruvate concentration due to limited D-pantothenate concentration had rather little effect on L-valine formation in *C. glutamicum ilvA* and *panB* mutants. The overexpression of L-valine synthesis genes in *aceE* and *pqo* mutants decreased the pyruvate concentration and shifted product pattern from pyruvate and L-alanine towards L-valine (Blombach et al. 2007b, 2008). Consequently, we can conclude that an increase in intracellular pyruvate concentration is essential but the strain's genetic background and cultivation conditions should not be neglected.

Besides pyruvate as L-valine precursor another significant compound essential for L-valine synthesis is cofactor NADPH that is used for the conversion of α-acetolactate by the AHAIR and for the synthesis of the primary amino group donor glutamate. A positive correlation between an increase in L-valine synthesis rate and the activity of G-6-PDH, NADPH producing enzyme from PPP, was observed. It might reflect an increase in NADPH generation in response to its increased utilisation in L-valine

synthesis. A similar correlation has also been previously reported for L-lysine producing *C. glutamicum* strains (Sonntag et al. 1995, Marx et al. 1997). This correlation was explained as a result of the decrease in feedback inhibition of the activity of G-6-PDH by NADPH as a consequence of an increase in this nucleotide utilization for L-lysine synthesis (Moritz et al 2000, Yokota and Lindley 2005). PPP represents the major source of NADPH supply in *C. glutamicum* (Cocaign-Bosquet et al. 1996).

However, the correlation between G-6-PDH activity and L-valine synthesis rate was not observed in all studied strains. No correlation between the increase in L-valine synthesis rate and intracellular concentration of NADPH was observed in any strains used in this study. Thus, our results suggested that NADPH concentration would not be a limiting factor in L-valine synthesis. Similarly Magnus et al. (2006) demonstrated that NADPH had only a modest influence on the flux through the L-valine pathway during glucose stimulus-response experiment in strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*). Recently it was shown that the deletion of *pgi* gene encoding phosphoglucose isomerase and thus redirecting the carbon flux towards the PPP resulted in a high increase in L-valine synthesis in *C. glutamicum aceE* and *pqo* mutants (Blombach et al. 2008, Bartek et al. 2010[a]). However, Marx et al. (2003) suggested that an increased NADPH pool due to certain genetic alterations and a subsequent increase in amino acid synthesis do not mean that the intracellular NADPH supply must have been limiting before redirection of the metabolic flux. The improved amino acid synthesis was explained by the fact that the whole metabolome is influenced in such a way that the pools of redox cofactors are more favourable for biosynthetic pathways which depend on NADPH as a cofactor (Marx et al. 2003).

Consequently, according to our results it can be concluded that precursor and cofactor supply for L-valine synthesis in L-isoleucine auxotroph *C. glutamicum* recombinant strains is an essential but not the limiting factor.

A correlation between the decrease in the cellular growth rate and the increase in AHAS and L-valine synthesis rate activity was observed in all studied strains. Thus the probable determinative role of AHAS in L-valine synthesis had to be elucidated. To have a more complete outlook on L-valine synthesis pathway, the activities of all

L-valine synthesis enzymes were also measured during the batch cultivation of *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*).

As it was already discussed here, the AHAS activity increased sharply after the decrease in the cellular growth rate. A slight increase was observed also in AHAIR and DHAD activities. The TmB activity showed almost no change during the cultivation. The only slight increase in AHAIR activity as compared to a sharp increase in AHAS activity could be explained by the fact that AHAIR is not inhibited by L-isoleucine, hence no effect of reduced feedback inhibition could be seen on this enzyme activity. Thus the increase in AHAIR activity could be partly due to derepressed transcription of *ilvBNC* operon under L-isoleucine limited conditions (Morbach et al. 2000) as AHAIR encoding gene *ilvC* is organized in operon together with *ilvBN* (Keilhauer et al. 1993). However, the *ilvBNC* operon of *C. glutamicum* is transcribed into 3 different transcripts *ilvBNC*, *ilvNC* and *ilvC* (Keilhauer et al. 1993), and *ilvC* transcript in *C. glutamicum* is present in a relatively large amount under various conditions. The latter observation was later explained by the fact that *ilvC* encodes not only AHAIR but also ketopantoate reductase, the enzyme of D-pantothenate pathway (Merkamm et al. 2003), and thus has a double function. It was also shown that NADPH has little influence on AHAIR reaction rate (Magnus 2007).

No previous detailed analyses on DHAD activity in *C. glutamicum* during the growth and L-valine synthesis were found in literature. Only recently in L-valine pathway flux modelling experiments Magnus (2007) suggested that DHAD is a non-limiting enzyme in L-valine synthesis in *C. glutamicum.* DHAD is not saturated by its substrate and very weakly inhibited by L-valine, therefore it could have little control of the flux through the pathway. It was even suggested that *ilvD* overexpression has to be removed to save the cell resources from overproducing the enzyme, which has a negligible influence on the L-valine flux (Magnus 2007). The minor role of DHAD in L-valine synthesis pathway was confirmed by Blombach et al. (2008), analysing L-valine synthesis in *C. glutamicum* Δ*aceE* harbouring different plasmids with different L-valine synthesis genes overexpressed. These data are in agreement with our conclusions that DHAD activity has a minor influence on L-valine pathway.

Somewhat contradictory are the data about TmB. Our results, showing a quite stable TmB activity throughout the growth, are in a good agreement with previously done metabolome flux analysis in D-pantothenate producing *C. glutamicum* strain

where the flux through α-ketoisovalerate (substrate for TmB) also remained constant during the fermentation (Chassagnole et al. 2002). Moreover, Radmacher et al. (2002) showed that *ilvE* overexpression on plasmid did not result in a significant increase in L-valine production. However, the *ilvE* overexpression enhanced L-valine production in *C. glutamicum* Δ*aceE* strain (Blombach et al. 2007[a], 2008). Besides, the L-valine pathway metabolite flux analysis showed that the net rate of the transamination from ketoisovalerate to L-valine is very low and there is an extremely high extracellular concentration of a-ketoisovalerate (Brik-Ternbach 2005), and it was suggested that TmB could have a significant impact on the increase in L-valine synthesis (Magnus 2007). Consequently the TmB role in L-valine synthesis could depend on certain strain genetic alterations that cause changes in the intracellular biochemistry.

Considering the literature data and our experimental results it was concluded that AHAS might have the main regulatory role in L-valine synthesis in L-isoleucine auxotrophic *C. glutamicum* strains: (i) it was the only L-valine enzyme strongly upregulated during the growth, (ii) only the increase in AHAS activity correlated with the increase in L-valine synthesis rate.

Hence it was very important to identify the parameters involved in AHAS activity regulation. It was already shown that the possible reason for the increase in AHAS activity in all studied strains could be the L-isoleucine limitation but not the pyruvate concentration. The analysis of L-isoleucine extracellular and intracellular concentrations during the batch cultivations confirmed that the increase in AHAS activity was related with the decrease in this amino acid extracellular and intracellular concentration.

L-isoleucine concentration became limiting not only due to the amino acid depletion but also due to the L-isoleucine uptake restriction by L-valine during the growth. All branched-chain amino acids are transported in cells with carrier BrnQ (Boles et al. 1993, Tauch et al. 1998), and this carrier has only slightly different affinities for L-isoleucine, L-valine and L-leucine (Ebbighausen et al. 1989). Therefore L-valine and L-isoleucine are competing for the uptake by BrnQ, especially under conditions when L-isoleucine concentration in the medium is considerably lower than L-valine concentration (Boles et al. 1993, Lange et al. 2003). Consequently, although there still is some L-isoleucine in the medium, the cell cannot use it because the increased L-valine concentration in the medium restricts L-

isoleucine uptake. Moreover, Boles et al. (1993) reported that external L-isoleucine could not be used by the cell, as long as it was added to the medium below a concentration of about 0.5 mM.

The positive effect of L-isoleucine limitation on AHAS activity was re-confirmed in short-term flask experiments where cells were exposed to differentially caused L-isoleucine limitation (due to the absence of L-isoleucine in the medium or due to the inhibition of L-isoleucine uptake in cell by L-valine). Thus it was emphasized that AHAS activity was strongly upregulated by L-isoleucine limitation. It was also obvious that the positive effect of L-isoleucine limitation on the AHAS activity was stronger than the inhibiting effect of high L-valine concentrations. Besides, it was demonstrated by Lange et al. (2003) that high L-valine concentrations do not inhibit the growth of *C. glutamicum* – the growth of *C. glutamicum* recombinant strain was not reduced in presence of 40 mM L-valine when dipeptide isoleucyl-isoleucine was supplied to the culture medium.

As it was previously shown that L-isoleucine inhibits AHAS activity (Leyval et al. 2003), the positive effect of L-isoleucine limitation on AHAS upregulation could be primarily associated with the reduced feedback inhibition of the enzyme. Secondly, the positive effect of L-isoleucine limitation on AHAS activity could be connected with the derepression of *ilvBN* expression (Morbach et al. 2000). It was demonstrated that *ilvB* expression was increased about 2-fold under conditions of growth limitation by any of the branched-chain amino acids (0.5 mM each) (Morbach et al. (2000). This allows us to conclude that after 8 h of cultivation, when L-isoleucine concentration in the medium decreases to 0.2-0.1 mM, the transcription of *ilvBNC* operon should be fully derepressed due to L-isoleucine limitation. Besides the L-valine production by L-isoleucine bradytrophic *C. glutamicum* strain was sharply reduced by the addition of 2mM L-isoleucine to the medium (Holátko et al. 2009). It was suggested that the increased L-valine production was more due to the growth-limitation by L-isoleucine than to the reduced flux through L-ioleucine synthesis pathway and increased availability of pyruvate.

However, the L-isoleucine limitation has not only a direct effect on the cell growth and enzyme activities – the growth limitation by an amino acid resulted in the stringent response induction in *C. glutamicum* cells (Ruklisha et al. 1993, Wehmeier et al. 1998, Tauch et al. 2001). And the stringent response could also cause AHAS

upregulation: it was previously reported that the stringent response induction was required for the derepression of transcription of *ilvBNC* operon under branched-chain amino acid limited conditions in *B. subtilis* (Eymann et al. 2002) and *E. coli* (Freundlich 1977, Tedin and Norel 2001).

Indeed, a sharp increase in intracellular ppGpp concentration (the signal molecule of the stringent response) was observed during 8-12 h of batch cultivation of strain *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*), indicating that the stringent response was launched. Therefore it was necessary to differentiate between the effect of L-isoleucine limitation and that of the stringent control induction on AHAS activity and L-valine synthesis in *C. glutamicum*. The role of *rel* gene presence on growth and L-valine synthesis was elucidated using *rel* proficient (rel^+) and *rel* deficient (rel^-) *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) strains.

The negative stringent control usually concerns reactions typical for growth and reproduction: an immediate reduction of the ribosomal RNA operon transcription and a massive reduction of the synthesis of stable RNA (rRNA and tRNA) (Cashel et al. 1996; Zhang et al. 2002), reduction of the synthesis of a certain mRNAs and an increase in the rate of protein degradation in bacterial cells (Lewin 2004). All these negative effects lead to changes in bacterial physiology, predominantly in the reduction of biomass formation and bacterial growth rate.

However, fed-batch cultivations of rel^+ and rel^- strains showed that their exponential growth under the conditions of L-isoleucine abundance as well as the growth rate reduction as a consequence of L-isoleucine depletion in the medium were comparable in both rel^+ and rel^- cells. The stringent response therefore did not induce an apparent effect on the cellular growth rate. Since the *rel*-dependent regulation of the nitrogen metabolism genes and the *rel*-independent regulation of the genes encoding ribosomal proteins in *C. glutamicum* have also been reported (Brockmann-Gretza and Kalinowski 2006), the mechanism of the growth control in this bacterium could be completely different from that reported for *E. coli, B. subtilis* or other bacteria (Cashel et al. 1996, Eymann et al. 2002, Wagner 2002). Moreover, it was reported that in some *C. glutamicum* recombinant strains the cellular growth even improved after *rel* gene deletion (Hayashi et al. 2006).

The results of short-term recultivations, similarly to those of fed-batch cultivations, showed that AHAS and AHAIR activity, as well as L-valine production

under the conditions of L-isoleucine limitation were increased in both *rel*$^+$ and *rel*$^-$ strains. Therefore, the stringent response was not found to be involved in enzyme upregulation in growth-limited *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*). Moreover, a distinct increase of AHAS and AHAIR activity in plasmid-less *rel*$^+$ and *rel*$^-$ *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 strains subjected to L-isoleucine limitation was also observed (data not shown). The strong upregulation of the transcription of genes encoding L-lysine synthesis under conditions of amino acid limitation in an L-lysine producing *C. glutamicum* strain was also found to be independent of stringent response induction (Hayashi et al. 2006). It should be noted that *rel*-independent AHAS and AHAIR upregulation in *C. glutamicum* is in contrast to the *rel*-dependent *ilvBN* expression upregulation in *E. coli* and *B. subtilis* (Cashel et al. 1996, Eymann et al. 2002, Tedin and Norel 2001).

However, the results of this study show that the presence of the *rel* gene is beneficial for L-valine synthesis by the late stationary phase of *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKA*ilvBNC*) cell culture. AHAS, TmB and particularly DHAD activity during the stationary growth phase was clearly higher in the *rel*$^+$ strain**.** These differences in enzyme activities in *rel*$^+$ and *rel*$^-$ strains can probably explain the fact that production of side amino acids was higher in *rel*$^-$ strain. L-valine synthesis rate and the final product concentration achieved by *rel*$^+$ strain was also higher than that achieved by *rel*$^-$ strain (75 mM and 50 mM, respectively or 33% lower L-valine production the *rel*$^-$ as compared to *rel* strain). It should be noted that the estimated biomass of *rel*$^-$ was only approximately 8% less than that of the *rel*$^+$ strain after 24-h cultivation (11.5 and 12.5 g l^{-1} respectively). Consequently, it can be concluded that *rel* gene has another, still unexplained role in L-valine synthesis regulation which should be further investigated, especially bearing in mind the fact that induction of this response by DL-serine hydroxamate only caused a stronger expression of genes involved in the synthesis of L-histidine and L-serine (Brockmann-Gretza and Kalinowski 2006).

As the presence of the *rel* gene had a positive effect in maintaining ATP-synthesis-coupled respiration and in minimizing ATP-synthesis-uncoupled respiration in cells subjected to L-isoleucine starvation, it may be assumed that that the positive effect of the stringent response on L-valine synthesis may result from its positive

effect in reducing oxidative stress. Since an increase in cyanide-resistant respiration in *C. glutamicum* has previously been reported to result from superoxide generation in cells (Shvinka et al. 1979), the comparatively low cyanide-resistant respiration could be an indication of a low oxidative stress in *rel*$^+$ cells.

On the other hand, it can be presumed that *rel* gene could potentially be involved in L-valine exporter expression regulation in *C. glutamicum*. The functioning of the possible regulator Lrp (leucine-responsive regulatory protein) is essential for the BCAAs export in *C. glutamicum,* suggesting an activating role for the expression of the carrier genes (Kennerknecht 2003). But the expression of the *lrp* gene on its turn was stimulated by ppGpp in *E. coli* (Landgraf et al. 1996, Park et al. 2007). Although the interconnection between *rel* and *lrp* has not yet been studied in *C. glutamicum,* it could be suggested that the stringent response elements could positively regulate BCAAs export.

Taken together, AHAS upregulation, resulting from L-isoleucine limitation, was established as a major factor enhancing L-valine production in L-isoleucine auxotrophic *C. glutamicum* recombinant strains. The stringent response was not shown to be directly involved in the enzyme and L-valine synthesis upregulation in this strain. Precursor supply is an essential but not the first limiting factor in L-valine synthesis in these strains.

It was shown that L-valine intracellular concentration could reach very high values as compared to extracellular values, even up to 250 mM (strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1*ilvBNCD*)). Therefore, the export of L-valine could be recognized as one of the bottlenecks for the increase in L-valine overproduction and could be stated as a new target for the improvement of L-valine overproduction. Magnus (2007) in L-valine synthesis pathway flux modelling experiment had also suggested that BCAAs translocase activity is the most limiting step in L-valine pathway. Export was identified as the most limiting step in L-isoleucine production (Morbach et al. 1996) as well. It has recently been reported that Lrp positively regulates the expression of the *ygaZH* genes encoding the L-valine exporter in *E. coli* and the L-valine production was synergistically improved by co-overexpression of the *lrp* and *ygaZH* genes (Park et al. 2007). Since Lrp is also essential for the BCAAs

export in *C. glutamicum*, it could be a new target for improvement of L-valine producers.

However, the state of art is to maintain high L-valine synthesis activity of cells under conditions of long-term L-valine production in bioreactor. In order to maintain a balance between the growth and L-valine synthesis, and to maintain the essential precursor supply and enzyme activities to achieve L-valine overproduction during fed-batch cultivations, two methods were developed: (i) maintenance of cellular growth during the stationary phase, (ii) maintenance of the restricted energy generation.

(i) It was found that despite of high AHAS activity, L-valine synthesis rate decreased during late stationary phase when cellular growth rate decreased below 0.05 h^{-1}. To avert the decrease in the growth rate and in the L-valine synthesis rate during the stationary phase, L-isoleucine pulse (0.22 mM) was added to the medium in the bioreactor. The increase in G-6-PDH activity and NADPH concentration due to L-isoleucine pulse allowed concluding that the maintenance of the cellular growth rate was important to maintain sufficient NADPH generation by PMP functioning, thus eliminating this nucleotide limit for L-valine synthesis by the stationary growth phase cell culture. Usage of this method allowed maintaining L-valine synthesis rate about 0.074 g val g cdm^{-1} during the stationary phase.

(ii) The experiment results showed that the decrease in cellular growth rate and energy generation in cells enhanced L-valine synthesis. It was also previously shown that L-valine production could be increased under conditions of limited oxygen supply (Akashi et al. 1977). It was explained from the view point of amino acid biosynthesis – in the L-valine synthesis oxidative and reductive reactions cancel each other out, and L-valine synthesis is an energy-generating reaction without consumption of oxygen for effective ATP formation. It was reported that L-lysine production strongly depends on oxygen supply as well (Ensari et al. 2003). However, it was shown that severe oxygen limitation did not succeed in an increase in L-valine synthesis, and it was supposed that changes in oxygen supply should be subtler. Therefore another method to achieve an increased L-valine overproduction was to restrict the growth and energy generation by restricted aeration intensity and respectively by oxygen

availability. Besides, it is known that dissolved oxygen tension is one of the important technological parameters influencing fermentation process.

These experiments showed that it is important to balance the growth and L-valine synthesis rate: a high respiration activity and high growth rates led to a high biomass accumulation but a rather low L-valine synthesis activity. Whereas the induction of L-valine synthesis in an early growth phase due to lower aeration intensity did not allow achieving the maximum biomass concentration. It could be connected with the previously shown extracellular L-valine inhibition effect on the L-isoleucine uptake and subsequently on the growth rate. Therefore a strategy to balance the biomass and L-valine synthesis was developed: pO_2 was adjusted at 25±3 % during first 7 hours of cultivation (growth phase), then during the L-valine synthesis phase pO_2 was decreased to 17±3 %. Thus, the cell culture was allowed to grow, but later the growth and energy generation in cells was restricted and an increased L-valine synthesis was achieved. As a result x=8 g l^{-1} per 1.14 mM L-isoleucine and a 3-fold increase in q_P (0.148 instead of 0.046g val g cdm^{-1} h^{-1}), a 2-fold increase in L-valine productivity (from 0.410, if pO_2=35 ± 3 % to 0.826 g l^{-1} h^{-1}, if pO_2=17±3 %) and a 2-fold increase in obtained L-valine concentration (from 8 g l^{-1} 24 h, if pO_2=35 ± 3 % to 16 g l^{-1} 24 h, if pO_2=17±3 %) were achieved during fed-batch cultivation with decreased pO_2.

5. CONCLUSIONS

1. A transition in the bacterial growth rate to below maximum was found to be an optimum parameter of cellular physiology to increase the activity of L-valine synthesis rate by *C. glutamicum* ATCC 13032 recombinant strains during batch and fed-batch cultivations.

2. The increase in L-valine synthesis rate in growth restrained cell cultures correlated with the increase in acetoxydroxyacid synthase activity in all the investigated strains under bacth and fed-batch cultivation conditions.

3. The increase in the pyruvate intracellular concentration correlated with the increase in L-valine synthesis rate and acetoxydroxyacid synthase activity only in some strains when (i) L-isoleucine limited and (ii) oxygen non-limited conditions were maintained. It was an indication that the pyruvate concentration is not the first limiting factor for the increase in L-valine synthesis rate.

4. The increase in acetoxydroxyacid synthase activity correlated with the decrease in L-isoleucine extracellular and intracellular concentrations, indicating that the possible reasons for acetoxydroxyacid synthase upregulation could be L-isoleucine limitation or the stringent response induction.

5. The acetoxydroxyacid synthase activity increased similarly in stringent (rel^+) and relaxed (rel^-) strains under L-isoleucine limited conditions, thus it can be concluded that the reason for acetoxydroxyacid synthase upregulation is L-isoleucine limitation. The stringent response is not involved in this enzyme upregulation.

6. Similar exponential growth of rel^+ and rel^- cells under L-isoleucine sufficient, and similar growth rate downregulation under L-isoleucine limiting conditions confirmed that the L-isoleucine limitation was a condition responsible for the

growth rate downregulation, and the stringent response was not involved in the cellular growth control in *C. glutamicum*.

7. Although the stringent response is not involved in the acetoxydroxyacid synthase upregulation under L-isoleucine limited conditions, the induction of the stringent response provided an increased L-valine synthesis rate during the stationary growth phase of fed-batch cultivations.

8. Based on the estimated principles of the increase in L-valine overproduction, technologies to maintain increased L-valine synthesis rate in *C. glutamicum* cells were developed:
    (i) L-isoleucine supplementation in a small doses in the bioreactor during the late stationary growth phase provided the cellular growth rate above 0.05 h^{-1} and 2 -fold increase in L-valine synthesis rate (from q_P 0.035 in control culture to q_P 0.074 g g cdm^{-1} h^{-1});
    (ii) A decrease in the aeration intensity (from pO_2 25% to pO_2 17%) and energy generation in cells provided 3-fold increase in L-valine synthesis rate (from q_P 0.046 in control culture to q_P 0.148 g g cdm^{-1} h^{-1}.

6. ACKNOWLEDGEMENTS

I would like to express my appreciation to all those who have supported me and have taken part in the research reported in this thesis. First of all I would like to thank my scientific supervisor Dr. habil. biol. Maija Rukliša for believing in me, supporting me all the time and for her many valuable comments concerning the work. I would like to thank my colleague in the laboratory Gina Paegle for her great help with the experimental work and just for her great personality.

Many thanks to researcher Nina Galinina for the help with the cellular respiration activity measurements.

Thanks to Rita Scerbaka for the assistance in the amino acid estimation.

In particular I would like to thank the colleagues from the Institute of Microbiology in the Czech Academy of Sciences for a very warm welcome in Prague and their kind assistance with research literature. I am very grateful to Dr. Marek Prouza and Dr. Miroslav Patek for providing the strains used in this study and for valuable discussions. Special thanks to Jiri Holatko for his great help with the introduction of HPLC amino acid estimation method in our institute and valuable discusions.

Thanks to Dr. L. Eggeling from Institute of Biotechnology, Juelich Research Centre, Germany for providing the strains used in this study. Thanks to Dr. H. Sahm and Dr. V. Wendisch for the very valuable time spent in Juelich Institute.

And last but not least my greatest thanks to my family for understanding and supporting me all the time. Special thanks to my sister for her great help with the language.

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