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# Branched-Chain Amino Acids

**Keisuke Yamamoto, Atsunari Tsuchisaka, and Hideaki Yukawa**

**Abstract** Branched-chain amino acids (BCAAs), viz., L-isoleucine, L-leucine, and L-valine, are essential amino acids that cannot be synthesized in higher organisms and are important nutrition for humans as well as livestock. They are also valued as synthetic intermediates for pharmaceuticals. Therefore, the demand for BCAAs in the feed and pharmaceutical industries is increasing continuously. Traditional industrial fermentative production of BCAAs was performed using microorganisms isolated by random mutagenesis. A collection of these classical strains was also scientifically useful to clarify the details of the BCAA biosynthetic pathways, which are tightly regulated by feedback inhibition and transcriptional attenuation. Based on this understanding of the metabolism of BCAAs, it is now possible for us to pursue strains with higher BCAA productivity using rational design and advanced molecular biology techniques. Additionally, systems biology approaches using augmented omics information help us to optimize carbon flux toward BCAA production. Here, we describe the biosynthetic pathways of BCAAs and their regulation and then overview the microorganisms developed for BCAA production. Other chemicals, including isobutanol, i.e., a second-generation biofuel, can be synthesized by branching the BCAA biosynthetic pathways, which are also outlined.

**Keywords** Branched-chain amino acids (BCAAs), *Corynebacterium glutamicum*, *Escherichia coli*, Feedback inhibition, Isobutanol, L-Isoleucine, L-Leucine, L-Valine, Metabolic engineering, Transcriptional attenuation

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**Contents**



# 1 Introduction

Branched-chain amino acids (BCAAs), namely, L-isoleucine, L-leucine, and L-valine, are essential amino acids [1] that are not synthesized in mammals, but have critical roles in physiological functions and metabolism [2]. BCAAs are used in dietary products, pharmaceuticals, and cosmetics and serve as a precursor of antibiotics and herbicides. Moreover, they are expected to play a leading role in future feed additives [3]. As other amino acids, BCAAs have been manufactured by fermentation using mutated or metabolically engineered microorganisms that originated from *Corynebacterium glutamicum* or *Escherichia coli* [4, 5]. The amount of the annual production of L-isoleucine, L-leucine, and L-valine in 2001 was approximately 400, 500, and 500 tons, respectively [6, 7], and has been increasing continuously. For example, the nonfeed market for L-valine reached around 1,000–1,500 tons per annum with an estimated 5–8 % annual increase [8].

Historically, BCAAs were produced chemically, and their enantiomers were separated enzymatically after chemical derivatization or via chromatographic separation followed by crystallization. However, as the demand for BCAAs increased, fermentative methods gathered attention for economic reasons as well as from an environmental perspective. In the early stages, most BCAA production strains were isolated by random mutagenesis. However, the random mutagenesis approach distributes genetic alterations throughout the chromosome, which are difficult to identify and may cause unexpected effects. Recently, amino acid-producing strains have been developed by rational genetic manipulation to avoid this problem. Highly productive strains can be created in a genetically defined manner, e.g., by specifically overexpressing biosynthetic genes responsible for a target amino acid. Systems metabolic engineering, which analyzes metabolism in a genome scale [9], also helps us to optimize carbon flux toward the biosynthesis of a target amino acid [10].

In this chapter, we will describe the biosynthetic pathways of BCAAs and their regulation in microorganisms, in particular, *C. glutamicum*, which is a nonpathogenic Gram-positive bacterium of the family Actinomycetes and is widely used for the industrial production of amino acids and nucleotides [11]. The development of

microbial strains for production of BCAAs will then be outlined. Finally, the extrapolated application of the BCAA biosynthetic pathways to other chemicals, including isobutanol, i.e., a second-generation biofuel, will be shown.

## 2 Regulation of BCAA Biosynthesis

The biosynthetic pathways of BCAAs of *C. glutamicum* are summarized in Fig. 1. *C. glutamicum* has one acetohydroxy acid synthase (AHAS) encoded by *ilvBN* [12], which catalyzes the initial step of the BCAA biosynthesis. AHAS produces

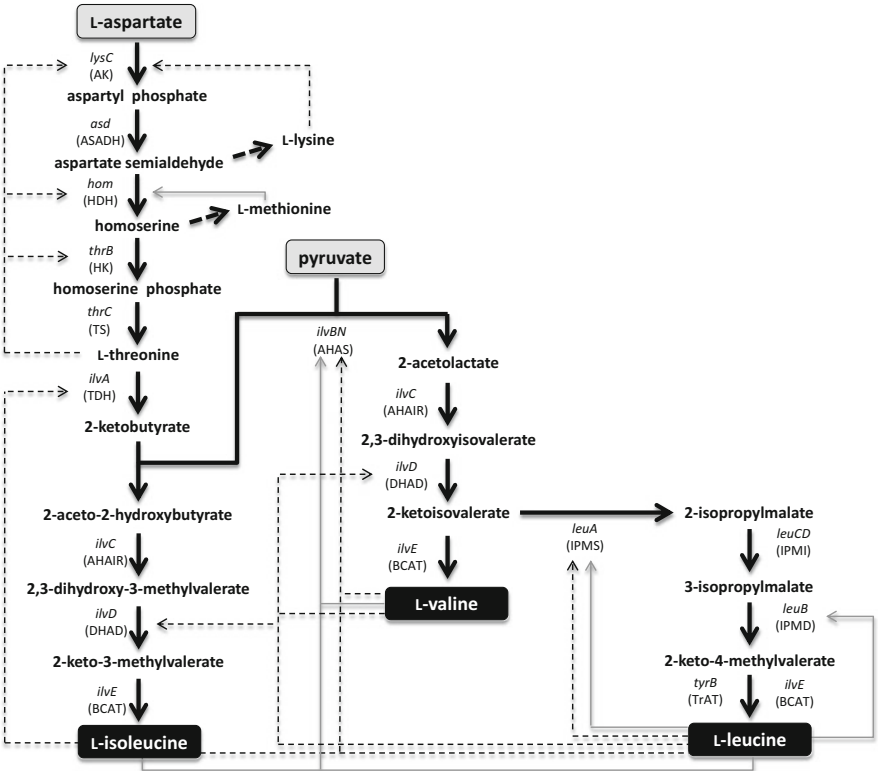


**Fig. 1** The BCAA biosynthetic pathways and their regulation in *C. glutamicum*. The genes and enzymes are shown in *italic font* and in *parentheses*, respectively. *Dotted lines* and *gray lines* indicate feedback inhibition and transcriptional attenuation, respectively. Abbreviations: *AHAIR* acetohydroxy acid isomeroreductase, *AHAS* acetohydroxy acid synthase, *AK* aspartate kinase, *ASADH* aspartate semialdehyde dehydrogenase, *BCAT* branched-chain amino acid aminotransferase, *DHAD* dihydroxy acid dehydratase, *HDH* homoserine dehydrogenase, *HK* homoserine kinase, *IPMD* isopropylmalate dehydrogenase, *IPMI* isopropylmalate isomerase, *IPMS* isopropylmalate synthase, *TDH* threonine dehydratase, *TrAT* tyrosine-repressible transaminase, *TS* threonine synthase

2-acetolactate and 2-aceto-2-hydroxybutyrate for the L-leucine/L-valine and L-isoleucine biosynthesis, respectively. The starting materials for this reaction are pyruvate and 2-ketobutyrate. Pyruvate is provided from the glycolytic pathway, while 2-ketobutyrate is synthesized from L-threonine by threonine dehydratase (TDH) encoded by *ilvA*. TDH is feedback inhibited by L-isoleucine [13], although the activity can be restored by L-valine [14]. AHAS consists of large and small subunits, which are encoded by *ilvB* and *ilvN*, respectively. The small subunit of AHAS is responsible for the multivalent regulation by all three BCAAs [12]. Unlike *C. glutamicum*, some bacteria have several isoforms of AHAS. For instance, *E. coli* has three isozymes, AHAS I, II, and III [3], which are encoded by *ilvBN*, *ilvGM*, and *ilvIH*, respectively. The expression of these genes is regulated differently, and *ilvGM* is attenuated by all BCAAs, while *ilvBN* is affected only by L-leucine and L-valine [15]. The activity of AHAS I and III is inhibited strongly by L-valine and weakly by L-isoleucine, while L-leucine has no effect [16]. *E. coli* is very sensitive to L-valine, and its growth is inhibited at an extremely low concentration [17].

The *ilvC* gene encodes acetohydroxy acid isomeroreductase (AHAIR) and is transcripted as an operon with the *ilvBN* genes [18] in *C. glutamicum*. AHAIR converts 2-acetolactate to 2,3-dihydroxyisovalerate in the L-valine and L-leucine biosynthesis and 2-aceto-2-hydroxybutyrate to 2,3-dihydroxy-3-methylvalerate in the L-isoleucine biosynthesis by using NADPH as a cofactor. The expression of *ilvBNC* operon is controlled by transcriptional attenuation, which is mediated by all BCAAs [19].

Dihydroxy-acid dehydratase (DHAD) encoded by *ilvD* [20] is the enzyme for the next biosynthetic step in which 2-ketoisovalerate and 2-keto-3-methylvalerate are formed from 2,3-dihydroxyisovalerate and 2,3-dihydroxy-3-methylvalerate, respectively. This enzyme is inhibited by either L-valine or L-leucine [21]. Transcriptional regulation of the *ilvD* gene is unknown.

Branched-chain amino acid transaminase (BCAT) or transaminase B encoded by *ilvE* [20] is the last player in the L-isoleucine and L-valine biosynthesis. This enzyme transfers the amine moiety of L-glutamate to 2-ketoisovalerate and 2-keto-3-methylvalerate to afford L-valine and L-isoleucine, respectively [22].

The specific pathway of the L-leucine biosynthesis starts from the reaction by isopropylmalate synthase (IPMS) encoded by *leuA*, which generates 2-isopropylmalate from 2-ketoisovalerate and acetyl-CoA. This enzyme in *C. glutamicum* is subjected to strong feedback inhibition, and its expression is also regulated by L-leucine [23]. Then, 2-isopropylmalate is isomerized to 3-isopropylmalate by isopropylmalate isomerase (IPMI). IPMI consists of large and small subunits, which are encoded by *leuC* and *leuD*, respectively. Next, 3-isopropylmalate is converted to 2-keto-4-methylvalerate by isopropylmalate dehydrogenase (IPMD), which is encoded by *leuB*. In *C. glutamicum*, *leuB* is strongly repressed by L-leucine [24], while the *leuABCD* genes form an operon in *E. coli*, and their expression is controlled by the transcriptional attenuation mediated by L-leucine [25]. As the other two BCAAs, L-leucine is formed from 2-keto-4-methylvalerate by the catalysis of BCAT, which is activated by the substrate itself

[26]. Additionally, the same reaction is catalyzed by tyrosine-repressible transaminase (TrTA) encoded by *tyrB* [27].

## 3 Production of BCAAs by Metabolically Engineered Microorganisms

In this section, we describe the BCAA-producing strains that have been developed to date. Table 1 shows the recent representative strains for BCAA production.

### *3.1 L-Valine*

Microbial production of L-valine was first reported by Udaka and Kinoshita [50] and Sugisaki [51] independently. Udaka and Kinoshita screened a large number of microorganisms, and the selected bacteria, namely, *Paracolobacterum coliforme* and *Brevibacterium ammoniagenes*, produced L-valine in 23 % molar yield from glucose. Sugisaki isolated *Aerobacter cloacae* and *A. aerogenes*, which produced L-valine in 20 % molar yield from glucose. These initial findings provoked a hunt for better L-valine producers, and auxotrophic mutants or amino acid analog-resistant mutants were included within the research scope. One example of the auxotrophic mutants is the isoleucine auxotrophic mutant of *Micrococcus glutamicus* [52], which produced more than 10 g/L L-valine when the culture was supplemented with a small amount of DL-isoleucine and DL-valine. An amino acid analog-resistant mutant accumulating L-valine was then found while validating a method to isolate amino acid-producing microorganisms using *E. coli* ATCC 4157 as a model microbe [53]. This L-valine-producing mutant was originated from a norvaline-resistant strain, which was further mutated to acquire L-leucine auxotrophy. The resultant strain accumulated more than 2 g/L L-valine. An amino acid analog-resistant mutant was also developed using *Serratia marcescens* [54]. Among several BCAA analogs tested, α-aminobutyric acid conferred mutants that were able to accumulate more than 8 g/L L-valine. An enzymatic analysis revealed that in these mutants, expression of AHAS was derepressed and/or AHAS was only weakly affected by feedback inhibition. Three L-glutamate-producing bacteria, *B. lactofermentum*, *C. acetoacidphilum*, and *Arthrobacter citreus*, were also led to L-valine production mutants using a histidine analog, 2-thiazolealanine [55]. The most efficient strain, one from *B. lactofermentum*, achieved 31 g/L production in 72 h. In this strain also, AHAS was desensitized from the feedback inhibition by L-valine as well as L-isoleucine and L-leucine. Moreover, the expression of this enzyme was partially derepressed [56]. An interesting example is the α-aminobutyric acid-resistant mutant of the biotin-auxotrophic *B. flavum* MJ-233 [57]. In the reaction using this mutant, carbon resources were converged to L-valine

**Table 1** The representative strains for production of BCAAs

| BCAA | Parent strain | Genotype | Plasmid | Reaction type | Final conc. (mM) | Yield (mol% of glucose) | Comment | Reference |
|---|---|---|---|---|---|---|---|---|
| L-Valine | *C. glutamicum* ATCC13032 | Δ*ilvA* Δ*panBC* | pJC1ilvBNCD | Flask | 92 | | | [20] |
| | | Δ*ilvA* Δ*panB ilvN*M13 | pECKAilvBNC | Flask | 130 | | | [28] |
| | | Δ*panB* *ilvN*M13 P-*ilvA*M1CG P-*ilvD*M7 P-*ilvE*M6 | | Flask | 136 | | | [29] |
| | | MP*ilvA* Δ*avtA* | pDXW-8-*ilvEBN*$^{r}$*C* | Fed-batch | 266 | 27 | 15 % saturation of DO | [30] |
| | | *atpG*DC* | pVKilvN53C | Flask | 96 | | | [31] |
| | | Δ*aceE* | pJC41ilvBNCE | Fed-batch | 210 | 60 | KOAc supplementation | [32] |
| | | Δ*aceE* Δ*pqo* | pJC41ilvBNCE | Fed-batch | 210 | 23 | KOAc supplementation | [33] |
| | | Δ*aceE* Δ*pqo* | pJC41ilvBNCE | Fed-batch | 301 | 20 | EtOH supplementation | [33] |
| | | Δ*aceE* Δ*pqo* Δ*sugR* | pJC41ilvBNCE | Fed-batch | 160 | 17 | | [33] |
| | | Δ*aceE* Δ*pqo* Δ*pgi* | pJC41ilvBNCE | Fed-batch | 412 | 75 | KOAc supplementation | [34] |
| | *C. glutamicum* R | Δ*ldhA* | pCRB-BN$^{GE}$C$^{TM}$ pCRB-DLD | Fed-batch | 1,470 | 63 | Oxygen deprivation condition | [35] |
| | | Δ*ldhA* Δ*pta* Δ*ackA* Δ*ctfA* *ilvN*$^{GE}$*C*$^{TM}$ *gapA*, *pyk*, *pfkA*, *pgi*, *tpi* | pCRB-BN$^{GE}$C$^{TM}$ pCRB-DLD | Fed-batch | 1,280 | 88 | Oxygen deprivation condition | [36] |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *C. glutamicum* ATCC13869 | Δ*aceE* Δ*ilvA* Δ*alaT* | pJYW-4-*ilvBNC*$_1$-*lrp*$_1$-*brnFE* | Fed-batch | 437 | 47 | | [37] |
| | *B. flavum* JV16 | *avtA*::*Cm* | | Fed-batch | 331 | 39 | | [30] |
| | *B. flavum* ATCC14067 | | pDXW-8-*ilvEBN*$^r$*C* | Fed-batch | 325 | 37 | High temperature (37°C) | [38] |
| | *E. coli* W3110 | *ilvH*(G41A C50T) $P_{tac}$-*ilvBN*, $P_{tac}$-*ilvGMED* Δ*ilvA* Δ*panB* Δ*leuA* Δ*aceF* Δ*pfkA* Δ*mdh* | pKBRilvBNCED pTrc184ygaZHlrp | Fed-batch | 64 | 58 | | [39] |
| L-Isoleucine | *C. glutamicum* ATCC 21799 | | pGC77 (*hom*$^{dr}$, *thrB*, *ilvA*) | Flask | 114 | | | [40] |
| | | | pAPE18 (*hom*$^{dr}$, *thrB*, *tdcB*) | Flask | 30 | | | [41] |
| | *C. glutamicum* MH20-22B | *hom*(Fbr) × 3 copies *thrB* × 4 copies | pECM3-*ilvA* (H278R-L351S) | Flask | 96 | | | [13] |
| | *C. glutamicum* MH20-22B | | pECM3-*ilvA* (V323A) pEK-*hom*(Fbr)-*thrB* | Flask | 63 | | | [42] |
| | *C. glutamicum* YILW | Δ*alaT* | pXMJ19-*thrABC* | Fed-batch | 100 | | | [43] |
| | *C. glutamicum* JHI3-156 | | | Batch | 203 | 27 | Optimized DO and pH control | [44] |
| | | | pDXW-8-*ilvBNC1-ilvA1* | Fed-batch | 234 | 16 | | [45] |
| | | | pDXW-8-*lrp-brnFE* | Fed-batch | 205 | 17 | | [46] |

(continued)

**Table 1** (continued)

| BCAA | Parent strain | Genotype | Plasmid | Reaction type | Final conc. (mM) | Yield (mol% of glucose) | Comment | Reference |
|---|---|---|---|---|---|---|---|---|
| | *C. glutamicum* WJ001 | | pDXW-8-*fusA-frr-ilvBNA-ppnk* | Fed-batch | 217 | 19 | | [47] |
| | *E. coli* K-12 TDH6 | | pVICLC80A (*thrA*BC*, *lysC**) pMWD5 (*ilvGMEDA**) | Flask | 94 | | | [48] |
| L-Leucine | *C. glutamicum* ATCC13032 | $P_{tuf}$-*leuA*_B018 Δ*leuA*::$P_{tuf}$-*leuA*_B018 Δ*ltbR*::$P_{tuf}$-*leuA*_B018 $P_{dap\text{-}L1}$-*gltA*, *ilvN*_fbr, Δ*iolR* | | Fed-batch | 152 | 26 | | [49] |

production when the medium lacked biotin and the bacteria did not grow [58]. This "living cell reaction" process using this strain allowed for accumulation of 300 mM L-valine within one day in 80 % molar yield from glucose with 96 % purity out of the total amino acids.

Genetically defined strains for L-valine production have been developed mostly by using *C. glutamicum* along with a few examples using other bacteria, including *E. coli*. The following mainly describes the strains derived from these two bacteria.

The general basic strategy in fermentative production of chemicals is to direct common intermediates for various end products solely to a target compound. In the case of L-valine, this can be achieved by deleting or repressing the transcription of *ilvA* and *panB* [20, 28–30, 59], which are the first genes for the L-isoleucine and D-pantothenate biosynthesis, respectively (Figs. 1 and 2). Limitation of D-pantothenate biosynthesis leads to reduction of CoA supply, which is an additional benefit for L-valine production because consumption of pyruvate by pyruvate dehydrogenase complex (PDHC) is suppressed due to the decreased concentration of the reaction partner [20].

Radmacher et al. [20] prepared *C. glutamicum* Δ*ilvA* Δ*panBC* whose biosynthesis of L-valine was strengthened by introduction of the plasmid containing *ilvBNCD* or *ilvBNCE*. The best strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1ilvBNCD) accumulated 92 mM L-valine along with a small amount of L-alanine (1.3 mM) in 48 h. Though AHAS was not desensitized from the feedback

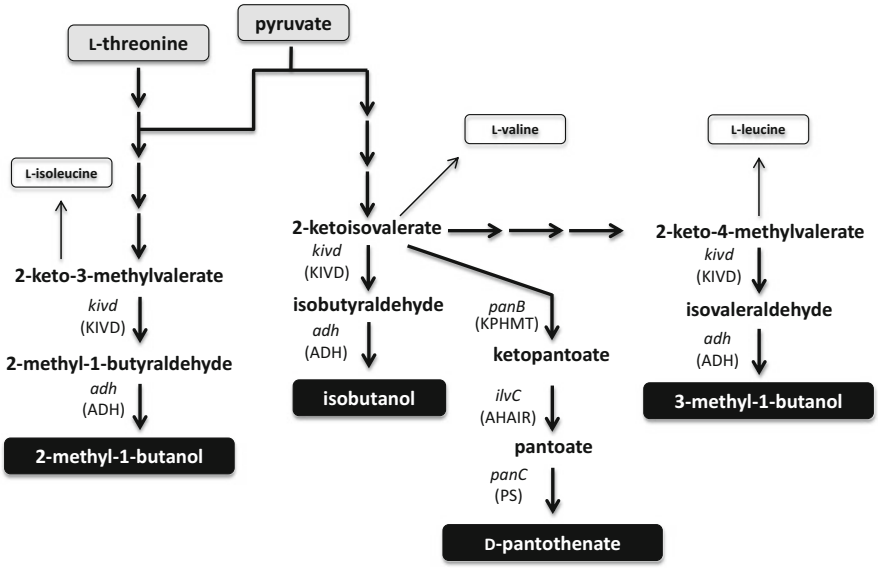


**Fig. 2** The biosynthetic routes branched from the BCAA biosynthetic pathways for production of isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and D-pantothenate. Abbreviations: *ADH* alcohol dehydrogenase, *AHAIR* acetohydroxy acid isomeroreductase, *KIVD* 2-ketoisovalerate decarboxylase, *KPHMT* ketopantoate hydroxymethyltransferase, *PS* pantothenate synthetase

inhibition by L-valine in this strain, it was able to produce L-valine efficiently. This is because the feedback inhibition of AHAS of *C. glutamicum* by L-valine is not very strong, and the maximum inhibition of the enzymatic activity does not exceed 50 % [12]. Nonetheless, desensitization of AHAS from the feedback inhibition was investigated [28]. The native gene of the regulatory subunit of AHAS (*ilvN*) was replaced with the feedback-resistant mutant *ilvN*M13, which was designed based on the precedent structural information on the feedback-resistant homologs of *E. coli* and *Streptomyces cinnamonensis*. This plasmid-free strain *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 produced 90 mM L-valine in the 48-h cultivation, while less than 40 mM L-valine formed in the culture of the parent strain *C. glutamicum* Δ*ilvA* Δ*panB*. L-Valine production by these strains was strengthened by a plasmid-carrying *ilvBNC*, and the resultant strain *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKAilvBNC) produced 130 mM L-valine. It should be mentioned that the effect of the mutation on *ilvN* was less significant in the strains with the plasmid-borne *ilvBNC*, and the parent *C. glutamicum* Δ*ilvA* Δ*panB* (pECKAilvBNC) was able to produce up to 120 mM L-valine.

Formation of L-alanine was a prevailing issue in the L-valine-producing strains [59]. Pyruvate is a common precursor for the biosynthesis of L-valine and L-alanine. Therefore, suppression of L-alanine formation is beneficial for L-valine production. Two genes, *alaT* or *avtA*, are responsible for the conversion of pyruvate to L-alanine, which encode aminotransferases using L-glutamate or L-valine as an amine source, respectively [59]. Deletion of either gene was performed on *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1ilvBNCD), and there was no detrimental effect on L-valine production in both cases. While the *avtA*-deficient mutant showed only slight decrease of L-alanine accumulation, the *alaT*-deficient mutant improved greatly, and L-alanine formation decreased to 0.16 mM from 1.2 mM in the parent strain. It should be mentioned that the effect of deletion of these genes may be dependent on strains, and in other cases, effectiveness of *avtA* deletion has been demonstrated as described below.

In place of the complete deletion of the side-path genes or strong overexpression of the rate-limiting enzymes for production of target compounds, their expression can be modified by tuning promoter activity. Holátko et al. [29] prepared *C. glutamicum ilvN*M13 Δ*panB* P-*ilvA*M1CG P-*ilvD*M7 P-*ilvE*M6, in which expression of *ilvA* was downregulated by the mutated promoter, while that of *ilvD* and *ilvE* was upregulated. This strain produced 136 mM L-valine in 48 h of flask fermentation. Modulation of promoter activities allows for overexpression of genes without using plasmids, which ensures genetic stability as well as no need for antibiotic markers. Additionally, the bradytrophic property is advantageous in that the strains do not require nutrition supplementation.

Recently, a strain with mixed strategies was reported [30], where expression of *ilvA* is partially limited by a mutated weak promoter, and production of L-alanine was suppressed by deletion of *avtA*. The resultant strain overexpressing the feedback-free L-valine biosynthetic genes, *C. glutamicum* MP*ilvA* Δ*avtA* (pDXW-8-*ilvEBN*$^{r}$*C*), produced 266 mM L-valine in 27 % molar yield from glucose. It is noteworthy that the side products, such as L-lactate and L-glutamate, were

controlled by maintaining the dissolved oxygen (DO) at 15 % saturation. The same strategy was applied to *B. flavum* JV16, which is an α-aminobutyric acid-resistant and Leu–Ile–Met-auxotrophic strain generated by random mutagenesis from *B. flavum* DSM20411. The resultant strain *B. flavum* JV16 *avtA*::*Cm* (pDXW-8-*ilvEBN*$^{r}$*C*) produced 331 mM L-valine in 39 % molar yield from glucose [30]. It should be mentioned that there is another example of an L-valine-producing strain using this subspecies of *C. glutamicum* [38]. *B. flavum* ATCC14067, which was transformed with the plasmid pDXW-8-*ilvEBN*$^{r}$*C*, was able to produce 325 mM L-valine in 37 % molar yield from glucose at elevated temperatures as high as 37°C after the 48-h fed-batch fermentation.

An alternative strategy for producing L-valine to deleting *ilvA* and *panB* was reported by Blombach et al. [32]. Deletion of *aceE*, which encodes the E1p subunit of PDHC, resulted in inability to grow solely on glucose, while acetate supplementation compensated for it [60]. Blombach et al. [32] investigated accumulation of organic acids and amino acids in the culture medium of this strain and found that it started to produce pyruvate (30–35 mM), L-alanine (25–30 mM), and L-valine (30–35 mM) after depletion of acetate. Pyruvate accumulated as a direct consequence of inactivation of PDHC, while L-alanine and L-valine were the drain-off compounds of pyruvate. When *ilvBNCE* was overexpressed using the plasmid, the resultant strain *C. glutamicum* Δ*aceE* (pJC4ilvBNCE) produced 210 mM L-valine in the overall 50 % molar yield from glucose along with 5 mM pyruvate in the fed-batch process. This strain is advantageous over the aforementioned Δ*ilvA* Δ*panB* strains because it does not require supplementation of L-isoleucine or D-pantothenate.

Blombach et al. [34] performed further improvement of this *aceE*-deficient strain. They deleted the *pqo* gene encoding pyruvate:quinone oxidoreductase (PQO), which converts pyruvate to acetate and carbon dioxide. This resulted in an increase of 30 % molar yield. It is likely that PQO in the combination with acetate kinase and phosphotransacetylase bypasses the PDHC reaction to provide acetyl-CoA when the cell density is high. Therefore, inactivation of PQO led to the increase of L-valine production by cutting off the supply of the carbon resources for growth purposes in the late phase. They continued to observe pyruvate in the culture of *C. glutamicum* Δ*aceE* Δ*pqo* (pJC4ilvBNCE), which indicated that L-valine production is limited by the downstream reactions from pyruvate to L-valine. They then tested to improve supply of NADPH. In the total reactions from glucose to L-valine, the downstream part (pyruvate to L-valine) requires two equivalents of NADPH at the AHAIR reaction as well as for regeneration of L-glutamate consumed by BCAT, whereas the glycolytic pathway provides only NADH, not NADPH. To compensate for the shortage of NADPH supply in L-valine production, they deleted *pgi* so that the carbon flux from glucose is directed to the pentose phosphate pathway, which produces 2 mol of NADPH from 1 mol of glucose. This strain, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*pgi* (pJC4ilvBNCE), achieved more than 400 mM L-valine excretion in the 75 % molar yield from glucose, and pyruvate was not observed anymore. From the viewpoint of carbon usage, the deletion of *pyc* encoding pyruvate carboxylase was also beneficial, and *C. glutamicum* Δ*aceE*

Δ*pqo* Δ*pgi* Δ*pyc* (pJC4ilvBNCE) reached 86 % molar yield, although the final concentration of L-valine was about 240 mM.

Addition of acetate inhibits production of L-valine during growth of the Δ*aceE* strains because acetate represses the genes of the phosphoenolpyruvate:sugar phosphotransferase system (PTS), *ptsG*, *ptsI*, and *ptsH* via the DeoR-type regulator, SugR, and prevents uptake of glucose [61–63]. Therefore, Blombach et al. [33] deleted the *sugR* gene to remove repression of the PTS genes. Indeed, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR* (pJC4ilvBNCE) consumed glucose five times faster than the parent strain and produced L-valine even in the growth phase in the presence of acetate, although the overall production was 40 % lower. Alternatively, they tested ethanol instead of acetate as the secondary carbon source to avoid the repression of the PTS genes. Under this condition, *C. glutamicum* Δ*aceE* Δ*pqo* (pJC4ilvBNCE) was able to produce L-valine during its growth phase. These strains are effective producers of L-valine, but they accumulated 14–26 mM L-alanine and pyruvate. This accumulation of the by-products may be overcome by supply of NADPH in the strategy mentioned above.

Recently, Chen et al. [37] prepared a strain, which lacked both *ilvA* and *aceE* and *alaT* from *C. glutamicum* ATCC13869 to direct as much pyruvate as possible for production of L-valine. They also overexpressed the *brnF* and *brnE* genes encoding the BCAA exporter and the *lrp* gene encoding the global regulator Lrp that activates the expression of *brnFE* in addition to the L-valine biosynthetic genes (*ilvBNC*). The resultant strain *C. glutamicum* ATCC13869 Δ*aceE* Δ*alaT* Δ*ilvA* (pJYW-4-*ilvBNC*$_1$-*lrp*$_1$-*brnFE*) produced 435 mM L-valine after a 96-h fermentation under the fed-batch condition.

Other strains of interest are the $H^+$-ATPase-defective strains [31], which are known to increase the intracellular concentration of pyruvate [64, 65]. The native *atpGDC* genes were replaced with the inactivated *atpG*DC* genes containing a single point mutation, and the resultant strain was transformed with the plasmid pVK7ilvN53C, which allows for overexpression of feedback-resistant AHAS as well as AHAIR. *C. glutamicum atpG*DC* (pVK7ilvN53C) produced 96 mM L-valine in 72 h under the flask-shaking condition.

A completely different strategy is to exploit oxygen deprivation conditions, where most of the glucose is expected to be utilized for product formation and not for growth in *C. glutamicum*. Hasegawa et al. [35] used the strain without the *ldhA* gene, which encodes lactate dehydrogenase and is responsible for the production of lactate, i.e., the main fermentation product under oxygen deprivation conditions. Mere overexpression of the L-valine biosynthetic *ilvBNCDE* genes did not result in the efficient L-valine production because of poor glucose uptake caused by the redox imbalance. They solved this issue by converting the cofactor dependence from NADPH to NADH through mutagenesis of AHAIR (*ilvC*$^{TM}$) and introduction of NADH-dependent exogenous leucine dehydrogenase in place of NADPH-dependent endogenous BCAT using the plasmid, pCRB-DLD. In addition, the feedback-resistant mutant of AHAS (*ilvBN*$^{GE}$) was overexpressed. The

resultant strain *C. glutamicum* R Δ*ldhA* (pCRB-BN$^{GE}$C$^{TM}$, pCRB-DLD) produced 1,470 mM[1] L-valine in 63 % molar yield from glucose in 24 h, and the concentration of L-valine reached 1,940 mM[1] in 48 h. This strain produced succinate as a major by-product and left room for improvement [36]. To minimize the carbon flux to succinate, the phosphoenolpyruvate carboxylase gene *ppc* was deleted. While succinate production was suppressed, this resulted in the elevated NADH/NAD$^+$ ratio. However, this redox imbalance was overcome by deletion of three genes, *ctfA*, *pta*, and *ackA*, associated with acetate synthesis, which produces excess NADH. Additionally, five glycolytic genes, *gapA*, *pyk*, *pfkA*, *pgi*, and *tpi*, were overexpressed. Moreover, L-alanine production was suppressed by deleting *avtA*. The resultant strain *C. glutamicum* R *ilvN*$^{GE}$*C*$^{TM}$, *gapA*, *pyk*, *pfkA*, *pgi*, and *tpi* Δ*ldhA* Δ*ppc* Δ*pta* Δ*ackA* Δ*ctfA* Δ*avtA* (pCRB-BN$^{GE}$C$^{TM}$, pCRB-DLD) produced 1,280 mM[1] L-valine in 88 % molar yield from glucose in the 24-h fed-batch fermentation.

Compared with *C. glutamicum*, development of the L-valine-producing strains of *E. coli* lags behind, which is probably because of the more complicated regulatory mechanisms for L-valine biosynthesis. However, using *E. coli* as a base strain is advantageous because of its rapid growth rate and rich genetic information. Early examples include the strains that acquire resistance against the feedback inhibition of AHAS III [66] or overexpress *ygaZH* encoding the L-valine exporter [67]. The strain with the mutation in isoleucine-tRNA synthetase [68] and the lipoic acid auxotroph with the inactivated H$^+$-ATPase [69] were also reported to accumulate L-valine. However, these strains were prepared by classical random mutagenesis and their genotypes cannot be defined. It was only recently that the rationally designed strain of *E. coli* was reported to produce L-valine [39]. In this example, Park et al. first removed the product regulation by disarming the feedback inhibition of AHAS III and removing the transcriptional attenuation of the *ilvBN* and *ilvGMEDA* operons by replacing their attenuator leader regions with *tac* promoters. Then the *ilvA*, *panB*, and *leuA* genes were knocked out to converge the carbon resources to L-valine production. Next, AHAS I, which has higher affinity to pyruvate than other two isozymes, was overexpressed as well as *ilvCED* by the plasmid pKBRilvBNCED. The strain was further improved by the plasmid pTrc184ygaZHlrp harboring *lrp* and *ygaZH* that encodes the positive regulator for the *ilvIH* operon and the L-valine exporter, respectively. In silico gene knockout simulation was then performed, and they identified three candidates to be deleted: *aceF*, *pfkA*, and *mdh*. When these genes were deleted, the resultant strain *E. coli ilvH* (G41A, C50T), P*tac-ilvBN*, P*tac-ilvGMED*, and Δ*ilvA* Δ*panB* Δ*leuA* Δ*aceF* Δ*pfkA* Δ*mdh* (pKBRilvBNCED, pTrc184ygaZHlrp) improved the yield and produced 64 mM L-valine in 58 % molar yield from glucose.

[1]The concentration values are corrected by the dilution factors caused by addition of ammonia solution to maintain the pH of the reaction solutions.

### 3.2 L-Isoleucine

An L-isoleucine producer was initially reported by Hayashibe and Uemura [70], which is an α-aminobutyric acid-resistant *B. subtilis* No. 14 that was isolated during the investigation of threonine metabolism and produced 4.3 g/L L-isoleucine. Screening of the α-aminobutyric acid-resistant microorganisms led to other L-isoleucine-producing strains, such as *A. aerogenes* IAM 1019 (2.4 g/L), *Pseudomonas aureofaciens* IAM 1001 (3.0 g/L), *S. marcescens* (2.7 g/L), and *Erwinia carotovora* E30 (1.3 g/L) [70]. Alternatively, D-threonine was used as a natural amino acid analog for the screening of the bacteria belonging to genera *Serratia* and *Pseudomonas* [71]. The most efficient strain among them, *S. marcescens* No. 1, produced more than 8 g/L L-isoleucine in a 40-h incubation. More microorganisms for L-isoleucine production were isolated using other BCAA analogs [72], including thiaisoleucine for *E. coli* [73, 74], *Salmonella typhimurium* [72, 75], and *Saccharomyces cerevisiae* [76], cyclopentaneglycine for *S. typhimurium* [75], glycyl isoleucine for *E. coli* [74], isoleucine hydroxamate for *S. marcescens* [77], and ketomycin for *Bacillus subtilis* [78]. As is the case for L-valine production, the "living cell reaction" process was effective for L-isoleucine production [58, 79, 80], which was performed using an α-aminobutyric acid-resistant *B. flavum* MJ-233 [57] to produce 200 mM L-isoleucine per day.

For rational metabolic engineering to enhance L-isoleucine production, it is important to address the fact that the biosynthetic pathway of L-isoleucine shares the genes and enzymes with those of L-valine and L-leucine (Fig. 1) and to converge the common intermediates toward the L-isoleucine synthesis. The specific issue for L-isoleucine production is the supply of L-threonine, which is one of the precursors for L-isoleucine biosynthesis. Additionally, removal of the tight feedback regulation for TDH (Fig. 1) is key to the efficient production of L-isoleucine.

As mentioned above, L-isoleucine production requires a supply of L-threonine. Therefore, the biosynthetic pathway of L-threonine and its regulation will be outlined briefly (Fig. 1). The biosynthesis of L-threonine starts from L-aspartate, and the pathway consists of five enzymatic reactions [81], which are catalyzed by aspartate kinase, aspartate semialdehyde dehydrogenase, homoserine dehydrogenase, homoserine kinase, and threonine synthase. *E. coli* has three aspartate kinase isozymes, I, II, and III, encoded by *thrA*, *metL*, and *lysC*, respectively, while *C. glutamicum* has only one aspartate kinase encoded by *lysC*. Aspartate kinases I and III of *E. coli* are under control of the feedback inhibition by L-threonine and L-lysine, respectively, whereas aspartate kinase II is not affected by feedback inhibition [82]. Instead, aspartate kinase II of *E. coli* is regulated by L-methionine through repression of *metBL* operon [83]. Aspartate kinase of *C. glutamicum* is subjected to the feedback inhibition by both L-lysine and L-threonine [13].

Homoserine dehydrogenase and homoserine kinase are encoded in the *hom* and *thrB* genes, respectively, in *C. glutamicum*. They form an operon and its expression is repressed by L-methionine [84]. Additionally, both enzymes are feedback inhibited by L-threonine [13]. In *E. coli*, *thrA* encodes a bifunctional enzyme that

works as homoserine dehydrogenase in addition to aspartate kinase [85]. This gene forms an operon with *thrB* and *thrC*, the latter of which encodes threonine synthase. Expression of the *thrABC* operon is controlled by transcriptional attenuation by L-threonine as well as L-isoleucine [82]. Moreover, the activity of homoserine dehydrogenase and homoserine kinase is feedback inhibited [82].

For L-isoleucine production, it is advantageous to use L-lysine-producing strains to supply L-threonine because the biosynthetic pathway of L-threonine shares a significant part with that of L-lysine. Colón et al. [40] reported accumulation of 114 mM L-isoleucine using an L-lysine-producing strain *C. glutamicum* ATCC 21799 (termed as *C. lactofermentum* in the original paper) by overexpressing the wild-type *ilvA*. Morbach et al. [13, 86] used another L-lysine-producing strain of *C. glutamicum* generated by random mutagenesis. They introduced multiple copies of the feedback-resistant *hom* and *thrB* in the chromosome or replaced the chromosomal native *lysC* and *hom* genes with the feedback-resistant ones. Along with overexpression of *ilvA*, the former strain produced 96 mM L-isoleucine in the batch culture and the latter 138 mM in the fed-batch culture. It should be mentioned that overexpression of *hom* was possible only after overexpression of *ilvA* because otherwise accumulation of L-threonine and L-homoserine caused instability of the strains [42]. Yin et al. [45] identified the feedback-resistant mutants of TDH and AHAS from the L-isoleucine-producing strain *C. glutamicum* JHI3-156. When the both feedback-resistant enzymes were overexpressed in the same strain, this resultant strain produced 234 mM L-isoleucine in the fed-batch condition. A comparative proteomic study on this strain further identified up- and downregulated proteins, which were related to cell growth, L-isoleucine biosynthesis, and stress response [87].

Introduction of exogenous genes is also a useful strategy. For example, an *E. coli*-derived *ilvA* was introduced to an L-threonine-producing *B. flavum* strain, a relative bacterium to *C. glutamicum*, to yield 153 mM L-isoleucine [88]. Wang et al. [43] introduced the *E. coli* K-12-originated *thrABC* genes to *C. glutamicum*. After deletion of *alaT*, the resultant strain produced 100 mM L-isoleucine along with low concentrations of L-lysine, L-alanine, and L-valine. Guillouet et al. [89] reported the advantage of the *tdcB* gene encoding the catabolic TDH of *E. coli* over the feedback-resistant *ilvA*. The strain with the *tdcB* gene accumulated four times more L-isoleucine than the one with the *ilvA* gene and yielded 30 mM L-isoleucine in the batch culture [41].

As is the case for L-valine, export of the accumulated product is important for efficient production of L-isoleucine. Therefore, overexpression of the global regulator Lrp and the BCAA exporter BrnFE was performed in *C. glutamicum* JHI3-156 to produce 205 mM L-isoleucine [46]. Recently, Zhao et al. [47] reported increased L-isoleucine production using *C. glutamicum* IWJ001, which was identified as an L-isoleucine-producing strain by random mutagenesis. They found that the biosynthetic enzymes for L-isoleucine were significantly upregulated when the *fusA* and *frr* genes, which encode ribosome elongation factor G and ribosome recycling factor, respectively, were overexpressed. Together with overexpression of *ilvA*,

*ilvB*, *ilvN*, and *ppnk* (a polyphosphate/ATP-dependent NAD kinase), the resultant strain produced 217 mM L-isoleucine in 72 h of fed-batch fermentation.

Optimization of the fermentation conditions is also an issue to be addressed. Peng et al. [44] optimized DO and pH of the fermentation conditions using *C. glutamicum* JHI3-156 (termed as *B. lactofermentum* in the original paper) and finally achieved 203 mM L-isoleucine in the batch culture.

*E. coli* is also the target of manipulation for L-isoleucine bio-production. Hashiguchi et al. [90] strengthened the downstream reactions by introducing the plasmid with the *ilvA*, *ilvGM*, *ilvD*, and *ilvE* genes to an L-threonine-producing *E. coli* K-12 mutant, and the resultant strain produced 78 mM L-isoleucine. This strain coproduced L-valine, but they solved this problem by introducing the gene of the feedback-resistant aspartate kinase III, which successfully reduced L-valine production and increased the final concentration of L-isoleucine to 94 mM [48].

### 3.3 L-Leucine

In the classical examples, accumulation of L-leucine was observed in the revertants of *S. marcescens* from the L-isoleucine auxotrophic mutant, which had been generated by resistance to α-aminobutyric acid [91]. Mechanistic investigations [91, 92] revealed that the resistance to α-aminobutyric acid was acquired by derepression of both L-isoleucine/L-valine and L-leucine biosynthetic enzymes. Interestingly, the reversion from the L-isoleucine auxotrophy was not due to desensitization of the feedback inhibition of TDH, but due to that of IPMS, which allowed for overproduction of L-leucine as well as supply of 2-ketobutyrate, i.e., a precursor for biosynthesis of L-isoleucine in place of TDH.

Another L-leucine-producing mutant was obtained from the glutamic acid-producing bacterium, *B. lactofermentum* [93]. This strain was screened from an L-methionine/L-isoleucine double auxotroph of *B. lactofermentum* 2256 using 2-thiazolealanine as an amino acid metabolism competitor. The optimization of the culture conditions allowed production of 30 g/L L-leucine [94, 95]. In this strain, IPMS is both desensitized and derepressed while AHAS remained intact [56]. Therefore, another screening was performed using β-hydroxyleucine, which obtained AHAS mutants desensitized from all of the BCAAs [96]. These strains showed improved productivity of 34 g/L L-leucine. A further campaign was conducted using high concentrations of D-α-aminobutyric acid, and a mutant with higher activities of AHAS and IPMS was isolated [97]. This mutant produced more L-leucine and showed a better L-leucine/L-valine ratio than the parent strain.

Mutants of *E. coli* have been developed for L-leucine production more recently. Some 4-azaleucine-resistant strains derived from *E. coli* K-12 were desensitized to the feedback inhibition of IPMS, and the most efficient strain produced 5.2 g/L L-leucine [25]. Alternatively, the L-isoleucine/L-valine double auxotroph with mutation in *ilvE* was isolated [98]. This strain was then supplemented with the plasmid to overexpress *tyrB*, which works only on the L-leucine biosynthesis among the

BCAAs. The resultant strain produced 2.7 g/L L-leucine with no detectable L-valine or L-isoleucine. Lowering the promoter activity of the *sucAB* gene encoding α-ketoglutarate dehydrogenase is likely to reduce the carbon flux into TCA cycle and decrease consumption of acetyl-CoA [99]. When this was combined with the feedback-free IPMS and the inactivated BCAT, the resultant strain produced 11.4 g/L L-leucine.

L-Leucine-producing strains were also found from amino acid auxotrophs of *C. glutamicum*. One of the best producers was the L-phenylalanine/L-histidine double auxotroph, which accumulated 16.0 g/L L-leucine. Another example is the *S*-(2-aminoethyl)-L-cysteine-resistant mutant of *C. glutamicum* [100], though this strain was found to be unstable and generated several types of revertants during the fermentation.

Recently, a genetically defined strain which produces L-leucine was reported [49]. This strain contains three copies of the $P_{tuf}$-*leuA*_B018 module in the chromosome. This gene, *leuA*_B018, encodes IPMS, which is disarmed from feedback inhibition by L-leucine. Moreover, in this module, the native promoter was replaced by the strong *tuf* promoter, which is free from transcriptional attenuation. Acetyl-CoA, i.e., another substrate for the IPMS reaction, was increased by replacing the native promoter of *gltA* (encoding citrate synthase) to $P_{dapA\text{-}L1}$. The transcriptional repressor-encoding gene *ltbR* was deleted to enhance expression of the downstream genes *leuBCD* for L-leucine production. Furthermore, mutations were introduced to the regulatory site of AHAS (encoded by *ilvN*) to be desensitized from feedback inhibition and increase the carbon flux toward L-leucine production. IolT1, which is regulated by IolR, catalyzes glucose uptake in a PTS-independent manner. Therefore, the *iolR* gene was also deleted to enhance glucose uptake. This strain, *C. glutamicum* $P_{tuf}$-*leuA*_B018 $P_{dap\text{-}L1}$-*gltA ilvN*_fbr Δ*ltbR*::$P_{tuf}$-*leuA*_B018 Δ*leuA*::$P_{tuf}$-*leuA*_B018 Δ*iolR*, produced up to 181 mM L-leucine in the culture solution along with precipitate of L-leucine after 72 h of the fed-batch fermentation.

## 4 Production of Chemicals Using the BCAA Biosynthetic Pathways

Recently, the instability of oil prices and environmental concerns has been driving the microbial production of biofuels. Such research includes ethanol fermentation as well as production of higher alcohols, which are expected to be next-generation biofuels due to their high energy density [101, 102]. Isobutanol is one representative example of such alcohols.

Isobutanol can be synthesized using the L-valine biosynthetic pathway (Fig. 2). 2-Ketoisovalerate, i.e., an intermediate of the L-valine biosynthesis, is a starting material for isobutanol production. It is first converted to isobutyraldehyde by 2-ketoisovalerate decarboxylase (KIVD), and then isobutanol is formed from the aldehyde by reduction using alcohol dehydrogenase (ADH). Like L-valine

production, overexpression of the genes encoding AHAS, AHAIR, and DHAD is effective to enhance availability of 2-ketoisovalarate for isobutanol production [103]. The bacterial strains developed for isobutanol production are reported for *E. coli* [104, 105], *B. subtilis* [106], and *C. glutamicum* [107], and the yields range between 74 and 297 mM. Although less efficient, *S. cerevisiae* is also used as a host for isobutanol production. The engineered strain overexpressing the L-valine biosynthetic genes accumulated 2.4 mM isobutanol [108], and the one whose L-isoleucine biosynthesis was eliminated produced 3.0 mM [109]. One of the difficulties in microbial production of isobutanol is that this alcohol is very toxic for microbes. To address this issue, Yamamoto et al. [110] performed isobutanol production in a growth-uncoupled manner under oxygen-deprived conditions. Moreover, they continuously extracted isobutanol from the aqueous reaction phase by layering oleyl alcohol and achieved as high as 981 mM of volumetric productivity.

In a similar manner to isobutanol, 3-methyl-1-butanol and 2-methyl-1-butanol can be produced from 2-keto-4-methylvalerate and 2-keto-3-methylvalerate, i.e., intermediates for the L-leucine and L-isoleucine biosynthesis, respectively, by the catalysis of KIVD and ADH (Fig. 2) [104, 111]. Such examples include the *E. coli* strain reported by Connor and Liao [112], where accumulation of 17 mM of 3-methyl-1-butanol was achieved when expression of the L-valine and L-leucine biosynthetic genes was enhanced to increase availability of 2-keto-4-methylvalerate for 3-methyl-1-butanol production. They also performed random mutagenesis and obtained an efficient strain that was able to produce 128 mM of 3-methyl-1-butanol in the biphasic fermentation using oleyl alcohol [113]. Cann and Liao [114] engineered *E. coli* to produce 2-methyl-1-butanol. In addition to strengthening the L-isoleucine biosynthetic pathway, the flux to L-threonine was optimized, and the resultant strain produced 14 mM of 2-methyl-1-butanol. Recently, production of isobutanol (17 mM), 3-methyl-1-butanol (8.5 mM), and 2-methyl-1-butanol (12 mM) was performed by the strains engineered for each alcohol with relevant genes from *S. cerevisiae* using *C. crenatum* as a parent strain [115].

Another important chemical for which the BCAA fermentative pathway can be applied is D-pantothenate. In *C. glutamicum*, it is produced from 2-ketoisovalerate by three enzymatic reactions catalyzed by ketopantoate hydroxymethyltransferase (KPHMT), AHAIR, and pantothenate synthetase (PS) [116] (Fig. 2). Hüser et al. [117] reported a D-pantothenate-producing strain. In this strain, *ilvA* was deleted, and the transcription of *ilvE* was attenuated to decrease the carbon flux to the competing BCAA biosynthesis. Additionally, overexpression of *ilvBNCD* was performed to increase availability of 2-ketoisovalerate as well as overexpression of *panBC* to direct more 2-ketoisovalerate to the D-pantothenate biosynthetic pathway. This strain produced 8 mM of D-pantothenate.

## 5 Conclusion

In this chapter, the biosynthetic pathway of BCAAs and its regulatory system were described, and the microbial strains engineered for BCAA production were summarized. The classical mutagenic strains provided a heritage of information about the complicated feedback inhibition and transcriptional attenuation in the BCAA biosynthesis. They now work as a guide for the rational design of BCAA-producing strains using advanced molecular biology techniques.

Generally, genetic modifications for producing target BCAAs are divided into four stages [3]: (1) to disarm feedback inhibition by the target BCAA, (2) to remove transcriptional attenuation by the target BCAA, (3) to minimize carbon flux to the competing pathways to reduce formation of by-products, and (4) to enhance expression of genes encoding biosynthetic enzymes of a BCAA to converge carbon resources as much as possible to production of it. Additionally, engineering of the export and import systems as well as modification of the transcriptional factors or the global regulators may be beneficial to improve production of the target amino acid further.

Disarming feedback inhibition is the most important step for efficient production of BCAAs because it has a very strong inhibitory effect on the microorganism growth and therefore the fermentative production of BCAAs. For example, the minimum inhibitory concentration of L-valine to the growth of *E. coli* K-12 is reported to be as low as 2 mg/L [17]. The complicated feedback inhibition system of the BCAA biosynthetic pathways was clarified by the early-stage studies on the auxotrophic strains and the amino acid analog-resistant strains. The key players of feedback inhibition are AHAS for all BCAAs, TDH for L-isoleucine, and IPMS for L-leucine, which can be now desensitized by rational mutation.

Production of BCAAs is also affected by transcriptional attenuation of the relevant genes. Derepression of the genes coding for AHAS is a common tactic because they are related to biosynthesis of all BCAAs. Removal of transcriptional attenuation of *leuA* is also important for L-leucine production. For L-isoleucine production, increasing supply of L-threonine by relieving transcriptional attenuation of the relevant genes may be beneficial. These can be performed by replacing the native promoters with others, e.g., *tac* promoter.

Minimization of carbon flux to competing pathways is required for efficient BCAA production. This can be achieved by deleting the corresponding genes. Pyruvate, which is the common intermediate for BCAA production, is also used for production of other competing by-products such as lactate, acetate, and succinate. Therefore, the genes responsible for production of these by-products are the target of deletion to increase availability of pyruvate for the BCAA biosynthesis. Additionally, for production of one of the BCAAs, the genes specifically relevant to the other two BCAAs are to be deleted, or their expression should be suppressed. For example, the *ilvA* gene, which is involved only in the L-isoleucine biosynthesis, is the target of deletion or attenuation for L-valine production.

Finally, overexpression of the genes responsible for biosynthesis of the target amino acid enhances its productivity. In particular, overexpression of AHAS, AHAIR, and DHAD, which catalyze the common reactions for all BCAAs, is effective for their production. It is beneficial to overexpress the *ilvA* and *leuA* genes for production of L-isoleucine and L-leucine, respectively, because they code for the enzymes responsible for their specific biosynthetic pathways. In addition, enhanced expression of the exporters of BCAAs as well as their positive regulators is also beneficial.

Currently, in silico studies of carbon flux simulation are emerging as a powerful tool to design strategies to maximize efficiency of biosynthetic pathways for production of the target compound [118, 119]. Application of these techniques will become an essential part of creating more sophisticated microbial strains, which can realize our goals.

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