An NADPH-auxotrophic *Corynebacterium glutamicum* recombinant strain and used it to construct L-leucine high-yielding strain

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Research Article

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Abstract

The NADPH-regeneration enzymes in *Corynebacterium glutamicum* were inactivated to construct an NADPH-auxotrophic *C. glutamicum* strain by gene knockout and gene replacement. The resultant NADPH-auxotrophic *C. glutamicum* XL-1 $\Delta ZMI_{Cg}::I_{Sm}$ (i.e., strain Leu-1) grew well in the basic medium only with gluconate as carbon source. Replacement of the native glyceraldehyde 3-phosphate dehydrogenase (NAD-GapDH_{Cg}) by NADP-GapDH_{Ca} from *Clostridium acetobutylicum* is an effective strategy for producing L-leucine in NADPH-prototrophic strain XL-1 and NADPH-auxotrophic strain Leu-1, whereas the L-leucine yield did not differ significantly between these strains (14.1 ± 1.8 g/L v.s. 16.2 ± 1.1 g/L). Enhancing the carbon flux in biosynthetic pathway by recombinant expression plasmid pEC-ABNCE promoted L-leucine production, but the shortage NADPH supply limited the L-leucine yield. The mutated promoters of *zwf* and *icd*_{Cg} were introduced into *C. glutamicum* with NADP-GapDH_{Ca} and pEC-ABNCE increased L-leucine yield (32.8 ± 1.7 g/L) and improved cell growth ($OD_{562} = 41.3 \pm 4.2$) because the resultant strain *C. glutamicum* XL-1 $\Delta MI_{Cg}::I_{Sm}$ G_{Cg}::G_{Ca} P_{zwf}-D1 P_{icd}-D2/pEC-ABNCE (i.e., strain Leu-9) exhibited the proper intracellular NADPH and NADH level. This is the first report of constructing an L-leucine high-yielding strain that reasonably supplies NADPH by optimizing the biosynthetic pathway of NADPH from an NADPH-auxotrophic strain.

Introduction

L-leucine is one of the essential amino acids for animal and human that needs to add into feed or food to meet the requirement of animal and human (Wang et al. 2019a). Given the important roles of L-leucine, it has been widely used as feed additive or nutritional supplement in feed or food industry (Luo et al. 2021; Wang et al. 2021). In addition, it is also widely used in chemical and pharmaceutical industry as cosmetic ingredient and medical intermediate, respectively (Ma et al. 2021; Wang et al. 2020). Although L-leucine would be produced by albuminolysis and enzymatic synthesis, microbial fermentation using *Corynebacterium glutamicum* as work-horse has become the mainstream method to produce L-leucine in industry at present because of the lowest environmental pollution and the best economic effect (Wang et al. 2020). Thus, the L-leucine producing strain with excellent fermentation performance is vital to increase the final titer and to reduce the production cost. To construct an L-leucine high-yielding strain, it not only needs to enhance the carbon flux in L-leucine biosynthetic pathway, but also needs to balance the supply of REDOX cofactor [i.e., NAD(H⁺) and NADP(H⁺)].

The effect of NADPH in amino acids production has been discussed by Xu et al. (2019), and Wang et al. pointed out that NADPH shortage is a rate-limiting factor for increasing L-leucine production (Wang et al. 2019b). As can be seen from Fig. 1, 2 molecules NADPH involve in L-leucine biosynthesis at acetohydroxyacid isomeroreductase (AHAIr)-catalyzed reaction and branched-chain amino acid transaminase (BCAT)-catalyzed reaction. In *C. glutamicum*, four enzymes were reported to involve in NADPH biosynthesis, i.e., glucose-6-phosphate dehydrogenase (Zwf), 6-phosphogluconate dehydrogenase (Gnd), NADP-dependent isocitrate dehydrogenase (NADP-Icd) and malic enzyme (MalE)(Fig. 1) (Hoffmann et al. 2021; Xu et al. 2019). However, the Zwf and Gnd in pentose phosphate (PP) pathway are the main enzymes for NADPH regeneration (Lindner et al. 2018). Increasing the activity of these key enzymes involved in NADPH biosynthesis is one of the most common strategies in constructing NADPH-dependent products high-producing strain (Xu et al. 2018). This is mostly achieved by increasing gene copies or replacing the native promoter by a strong promoter, or relieving the feedback regulation of enzyme (Li et al. 2016; Xu et al. 2018). Although these strategies have acquired certain positive results, the negative factor as the shadow follows the form. For example, enhancement the carbon flux in PP pathway by overexpression of Zwf- and Gnd-coding gene not only improves NADPH supply but also increases the release of CO₂ (Jiang et al. 2013; Yuan et al. 2021). Moreover, irrational supply of NADPH results in the excesses of intracellular NADPH, thus leading to more reactive oxygen species (ROS) production and to injure cells (Xu et al. 2019; Zhu et al. 2021). Therefore, how to optimize the NADPH-supplying pathway under no background noise is important for constructing NADPH-dependent products producing strains with excellent fermentation performance.

In 2018, the researchers in Arren Bar-Even's Lab from Max Planck Institute of Molecular Plant Physiology (Potsdam-Golm, Germany) constructed an *Escherichia coli* NADPH-auxotroph strain via inactivation of enzymes in NADPH biosynthetic pathway (Lindner et al. 2018), and used it as chassis strain to select formate dehydrogenase variants with high efficiency and specificity toward NADP⁺ (Calzadiaz-Ramirez et al. 2020). Refer to the above experimental considerations, therefore, the objective of this study was to try to construct an L-leucine high-producing strain based on optimizing NADPH-supplying pathway from *C. glutamicum* NADPH-auxotroph strain. The main methods of this study involved in three aspects: (1) constructing a *C. glutamicum* NADPH-auxotroph strain via inactivation of enzymes in NADPH biosynthetic pathway from an L-leucine producing strain *C. glutamicum* XL-1 (Wang et al. 2019b); (2) replacing the native NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (NAD-GapDH_{Cg}) by NADP-GapDH_{Ca} from *Clostridium acetobutylicum*; (3) rationally regulating the expression levels of Zwf-coding gene *zwf* via promoter engineering. As a result, a high-yielding strain *C. glutamicum* Leu-9 with constructed weak promoter of *zwf* was obtained, which produced 32.8 ± 1.7 g/L of L-leucine in 72-h shake flask fermentation. Here, we report an effective strategy to construct an L-leucine high-producing strain from an NADPH-auxotrophic strain by optimizing the biosynthetic pathway of NADPH for the first time. The design-based strategy for constructing L-leucine high-yielding strain reported here could serve as a general concept for breeding NADPH-dependent products high-yielding strains.

Materials And Methods

Strains, growth medium and culture conditions

Strains used in this study are listed in Table 1. The parental strain *C. glutamicum* XL-1 was an L-leucine producing strain constructed by Wang et al. (2019b). *E. coli* and *C. glutamicum* were cultivated in Luria-Bertani (LB) medium at 37°C and in LB-glucose (LBG) medium at 30°C, respectively (Xu et al. 2016). CgXII medium, consisting of (per liter) 42 g of 3-morpholinopropanesulfonic acid, 20 g of (NH₄)₂SO₄, 5 g of urea, 1 g of KH₂PO₄, 1 g of K₂HPO₄·3H₂O, 0.25 g of MgSO₄·7H₂O, 0.01 g of CaCl₂, 0.01 g of FeSO₄·7H₂O, 0.01 g of MnSO₄·H₂O, 0.01 g of ZnSO₄·7H₂O, 0.0002 g of NiCl₂·6H₂O, 0.0002 g of biotin and 0.00003 g of protocatechuic acid, (Keilhauer et al. 1993) was used for growth experiments. 36 g of glucose or the equivalent other carbon sources (calculating based on the energy of combustion among these carbon sources (Lindner et al. 2018)) were used as the carbon source. Appropriately, 50 µg/mL or 25 µg/mL kanamycin

(Kan) solution was used to screen the target plasmids and strains. In addition, 1 mmol/L isopropyl β -D-thiogalactopyranoside (IPTG) solution was used to induce gene overexpression.

Table 1
The main strains and plasmids used in this study

Strains and plasmids	Characters	Reference
C. glutamicum strains		
ATCC13032	Wild-type strain	Stratagene
XQ-9	<i>C. glutamicum</i> α -AB ⁺ 2-TA ⁺ SG ⁺ β -HL ⁺ Ile ⁻ Met ⁻ , a L-leucine producing strain derived from strain ATCC13032	(Wang et al., 2019b)
XL-1	Strain XQ-9 derivative with in-frame deletion of <i>ItbR</i>	(Wang et al., 2020)
Leu-1	An NADPH-auxotrophic strain derived from strain XL-1 with inactivated <i>Zwf</i> and <i>MalE</i> as well as replacement with NAD-dependent <i>Icd</i>	This study
Leu-2	The native NAD-dependent GapDH was replaced by NADP-dependent GapDH in strain Leu-1 chromosome	This study
Leu-3	The native promoter of gene <i>zwf</i> was replaced by promoter P_{zwf} -D5 in strain Leu-2 chromosome and harboring plasmid pEC-ABNCE	This study
Leu-4	The native promoter of gene <i>zwf</i> was replaced by promoter P_{zwf} -D1 in strain Leu-2 chromosome and harboring plasmid pEC-ABNCE	This study
Leu-5	The native promoter of gene <i>zwf</i> was replaced by promoter P_{zwf} -S6 in strain Leu-2 chromosome and harboring plasmid pEC-ABNCE	This study
Leu-6	The native promoter of gene <i>icd</i> was replaced by promoter P_{icd} -D2 in strain Leu-2 chromosome and harboring plasmid pEC-ABNCE	This study
Leu-7	The native promoter of gene <i>zwf</i> was replaced by promoter P_{icd} -S7 in strain Leu-2 chromosome and harboring plasmid pEC-ABNCE	This study
Leu-8	The native promoter of gene <i>zwf</i> was replaced by promoter P_{icd} -T3 in strain Leu-2 chromosome and harboring plasmid pEC-ABNCE	This study
Leu-9	The native promoters of genes <i>zwf</i> and <i>icd</i> were replaced by promoter P_{zwf} -D1 and P_{icd} -D2 in strain Leu-2 chromosome and harboring plasmid pEC-ABNCE	This study
Leu-10	The native promoters of genes <i>zwf</i> and <i>icd</i> were replaced by promoter P_{zwf} -D1 and P_{icd} -S7 in strain Leu-2 chromosome and harboring plasmid pEC-ABNCE	This study
Leu-11	The native promoters of genes <i>zwf</i> and <i>icd</i> were replaced by promoter P_{zwf} -D1 and P_{icd} -T3 in strain Leu-2 chromosome and harboring plasmid pEC-ABNCE	This study
Plasmids		
pEC-XK99E	Kan ^r , Expression vector with <i>pMB1</i> replicon	Stratagene
pEC-ABNCE	Kan ^r , pEC-XK99E derivative containing gene <i>leuA</i> , <i>ilvBN</i> , <i>ilvC</i> and <i>ilvE</i>	(Wang et al., 2019b)
pEC-ABNC ^{ME}	Kan ^r , pEC-ABNCE derivative integrated mutations into <i>ilvC</i> coding for amino acid exchanges S34G, L48E, and R49F	(Wang et al., 2019b)
pDXW-11	Kan ^r , <i>E. coli</i> - <i>C. glutamicum</i> shuttle probe vector	(Xu et al., 2011)
pDXW-11- P_M	400-bp insert with the gene <i>M</i> promoter region (gene <i>M</i> represented <i>zwf</i> , <i>gnd</i> , <i>icd</i> , <i>malE</i> and <i>gapA</i>)	This study
pK18 <i>mobsacB</i>	Integration vector	Stratagene
pK18 <i>mobsacB</i> / ΔP_{zwf}	Integration vector for deleting the promoter of <i>zwf</i>	This study
pK18 <i>mobsacB</i> / ΔP_{malE}	Integration vector for deleting the promoter of <i>malE</i>	This study
pK18 <i>mobsacB</i> / $\Delta icd_{Cg}::icd_{Sm}$	Integration vector for replacement of the ORF of <i>icd</i> _{Cg} gene by the ORF of <i>icd</i> _{Sm} gene	This study
pK18 <i>mobsacB</i> / $\Delta gapA::gapC$	Integration vector for replacement of the ORF of <i>gapA</i> gene by the ORF of <i>gapC</i> gene	This study
pK18 <i>mobsacB</i> / $\Delta P_{zwf}::P_{zwf-M}$	Integration vector for replacement of the promoter of <i>zwf</i> gene by the mutated promoter of <i>zwf</i> gene (i.e., P_{zwf} -D5, P_{zwf} -D1 and P_{zwf} -S6)	This study
pK18 <i>mobsacB</i> / $\Delta P_{icd}::P_{icd-M}$	Integration vector for replacement of the promoter of <i>icd</i> gene by the mutated promoter of <i>icd</i> gene (i.e., P_{icd} -D2, P_{icd} -S7 and P_{icd} -T3)	This study

Batch shake-flask fermentation was carried out in 500 mL shake flask with 50 mL of fermentation medium, and was referred to the methods described by Feng et al (2018). Inoculum was from a seed culture with $\Delta OD_{562} = 0.45\text{--}0.50$ (at a dilution of 25-fold), and the inoculation amount was 10%. The seed medium was prepared according to the previous reports described by Wang et al. (2020). And the fermentation medium was prepared according to the following formula (per liter): 130 g glucose, 25 g corn steep powder, 15 g $(\text{NH}_4)_2\text{SO}_4$, 15 g $\text{CH}_3\text{COONH}_4$, 2 g/L $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$, 2 g/L urea, 1 g KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.7 g/L L-methionine, 0.5 g/L L-glutamate, 0.06 g/L L-isoleucine, 160 $\mu\text{g/L}$ thiamine·HCl, 50 $\mu\text{g/L}$ biotin and 30 g/L CaCO_3 . Media were adjusted to pH7.3 with 20% (m/v) NaOH.

Construction of *C. glutamicum* recombinant strains

Plasmids and oligonucleotides used in this study are listed in Table 1 and Supplementary material (Table S1), respectively. Plasmid pEC-XK99E was used for gene expression, and plasmid pK18*mobsacB* was used for gene deletions and gene replacements in *C. glutamicum*. The detail methods used for gene modification (i.e., gene expression, gene deletion and gene replacement) were referred to the published method (Liu et al., 2021). To construct Zwf- and/or MalE-deficient strain, the promoters of gene *zwf* and/or *malE* were deleted. The cassettes of *gapC_{Ca}* with the *P_{gapA}* promoter, *rrnBT1T2* terminator and *EcoRI* endonuclease was optimized for expression in *C. glutamicum* and then synthesized by GENEWIZ (Suzhou), Inc. (Suzhou, China). The detail processes of plasmid construction were listed in Supplementary material (Figure S1). For modifying promoter, fragments (~ 400 bp) carrying promoters of the genes *zwf*, *gnd*, *malE*, *gapA* and *icd* were amplified from the chromosome of *C. glutamicum* XL-1 by PCR with corresponding primers (Table S1). The PCR products after gel purification were digested by the corresponding restriction endonucleases and cloned in the plasmid pDXW-11 (Xu et al., 2011). The resultant plasmids were transferred into *C. glutamicum* by electroporation, and the target recombinants were selected referring to the published method (van der Rest et al. 1999).

Preparation of crude enzyme and enzyme activity assays

The cells in the logarithmic growth were collected after centrifugation for 10 min at $5000\times g$, and then were disrupted by sonication. Following, fragmented liquid was centrifuged by centrifuge at 4°C and $10000\times g$ for 30 min, and then the cell-free supernatants were used as crude enzyme to determine the enzyme activities. Protein concentrations were analyzed using the Bradford Protein Quantification Kit (Vazyme, Nanjing, China) with bovine serum albumin as standard. The enzyme activity assays were done in triplicate, and the analytical methods of Zwf, Gnd, IDH, MalE, GapDH and chloramphenicol acetyltransferase (CAT) were based on the protocol of Tran et al. (2021), Cardinali-Rezende et al. (2020), Wang et al. (Wang et al., 2015), Hörl et al. (2021), Xu et al. (2014) and Holátko et al. (2009), respectively.

Quantification of intracellular pyridine nucleotides

The intracellular pyridine nucleotides (i.e., NADH/NAD and NADPH/NADP) of cells cultivated for 36 h were extracted referring to the published method (Faijes et al. 2007), and the concentration was detected using NAD/NADH Quantification Colorimetric Kit or NADP/NADPH Quantification Colorimetric Kit (BioVision, Inc., Milpitas, CA) based on the manufacturer's instructions, respectively.

Analytical methods

A sample was taken from the shake flasks at the right time. The sample was used to analyze cell growth using a spectrophotometer at 562 nm after an appropriate dilution. In addition, glucose concentration was determined using an SBA-40E immobilized enzyme biosensor (Shandong, China). The concentration of L-leucine was determined by high performance liquid chromatography (HPLC) using Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA) according to the procedure described by Wang et al. (2020).

Results

Construction and characterization of an NADPH-auxotrophic *C. glutamicum* strain

In *C. glutamicum*, there are four enzymes that are reported to involve in NADPH regeneration (Fig. 1) (Hoffmann et al. 2021; Xu et al. 2019). In order to construct an NADPH-auxotrophic *C. glutamicum* strain, we aimed to inactivate the Zwf and the MalE as well as to replace the native NADP-Icd_{Cg} by NAD-Icd_{Ca} from *S. mutans*. In this study, the Gnd was not inactivated because the activity of Gnd was controlled by the availability of 6-phosphogluconate, which was the product in Zwf-catalyzed reaction (Fig. 1). In addition, the strain with the activated Gnd can maintain the cell growth during using gluconate as carbon source because gluconate can convert to 6-phosphogluconate without Zwf participation (Lindner et al. 2018). The cell growth of Zwf-deficient strain (i.e., *C. glutamicum* XL-1 ΔZ) was significantly decreased during growth on most carbon source except on gluconate, whereas the cell growth of MalE-deficient strain (i.e., *C. glutamicum* XL-1 ΔM) had little change during growth on the test carbon sources except on succinate (Fig. 2a-c). It is worth noting that replacement of the native NADP-Icd_{Cg} by NAD-Icd_{Sm} hardly affected growth on all carbon sources (Fig. 2d), but disruption of NADP-Icd_{Cg} severely inhibited growth on most other carbon sources except on succinate (Fig. 2e). Next, we integrated the above gene modifications with the disruption of MalE and/or replacement with NAD-Icd_{Sm}. As can be seen from Fig. 2b, f, g, further disruption of MalE in strain XL-1 ΔZ was not significantly impaired cell growth on any of the carbon sources, but further replacement with NAD-Icd_{Sm} in strain XL-1 ΔZ almost abolished cell growth on most carbon source except on gluconate. Deletion of *zwf* and *malE* as well as replacement with *icd_{Sm}* (i.e., *C. glutamicum* XL-1 $\Delta Z\text{MI}_{Cg::I_{Sm}}$, also known as strain Leu-1) completely abolished cell growth on most carbon source except on gluconate (Fig. 2h). Different from the cell growth, the L-leucine yield of these recombinant strains was obviously decreased with modification of the enzyme involved in NADPH regeneration except the strain *C. glutamicum* XL-1 ΔM during cultivated in fermentation medium (Table 2). Thus, we selected strain Leu-1 as NADPH-auxotrophic *C. glutamicum* strain to optimize NADPH-supplying pathway for constructing L-leucine high-producing strain.

Table 2
Cell weight, L-leucine yield and content of intracellular pyridine nucleotides of recombinant and original strains in the fermentation medium

<i>C. glutamicum</i>	OD ₅₆₂	L-leucine ^a	NADPH ^b	NADP ⁺ ^b	NADH ^b	NAD ⁺ ^b
XL-1	39.5 ± 2.7	15.3 ± 1.2	3.74 ± 0.35	4.62 ± 0.23	4.27 ± 0.43	16.76 ± 1.55
XL-1 ΔZ	27.2 ± 1.8	ND	0.93 ± 0.10	1.86 ± 0.12	-	-
XL-1 ΔM	40.3 ± 5.2	14.5 ± 1.6	3.51 ± 0.18	4.67 ± 0.47	-	-
XL-1 ΔI _{Cg} ::I _{Sm}	37.2 ± 2.3	8.6 ± 1.5	2.69 ± 0.21	4.54 ± 0.26	-	-
XL-1 ΔZ ΔM	25.9 ± 2.5	ND	0.74 ± 0.08	1.65 ± 0.21	-	-
XL-1ΔZ ΔI _{Cg} ::I _{Sm}	3.5 ± 0.2	ND	0.07 ± 0.01	0.56 ± 0.04	-	-
Leu-1	-	-	-	-	-	-
Leu-2	34.6 ± 3.2	16.2 ± 1.1	4.48 ± 0.34	4.67 ± 0.50	2.23 ± 0.24	14.89 ± 1.19
XL-1 G _{Cg} ::G _{Ca}	26.4 ± 3.7	14.1 ± 1.8	5.25 ± 0.29	4.33 ± 0.27	2.16 ± 0.16	14.78 ± 1.26
Leu-2/pEC-ABNCE	40.7 ± 3.4	26.3 ± 1.7	3.06 ± 0.17	4.94 ± 0.24	2.75 ± 0.25	15.28 ± 1.12
Leu-2/pEC-ABNC ^{ME}	30.4 ± 3.7	22.6 ± 2.3	4.24 ± 0.38	4.76 ± 0.43	2.44 ± 0.13	14.35 ± 1.43
Leu-3	35.7 ± 4.0	24.8 ± 2.7	4.08 ± 0.24	4.61 ± 0.31	2.85 ± 0.21	15.01 ± 1.62
Leu-4	37.2 ± 2.2	28.6 ± 2.4	3.57 ± 0.37	4.63 ± 0.19	2.97 ± 0.17	14.85 ± 0.87
Leu-5	38.9 ± 2.6	26.9 ± 2.1	3.15 ± 0.19	4.79 ± 0.36	2.79 ± 0.28	15.23 ± 1.70
Leu-6	45.9 ± 4.6	24.2 ± 2.2	3.53 ± 0.28	4.65 ± 0.42	4.32 ± 0.23	16.62 ± 1.02
Leu-7	48.1 ± 3.9	23.6 ± 2.4	3.51 ± 0.33	4.65 ± 0.35	4.57 ± 0.47	16.32 ± 1.43
Leu-8	52.6 ± 6.4	22.3 ± 1.3	3.37 ± 0.26	4.48 ± 0.29	5.08 ± 0.44	16.93 ± 1.71
Leu-9	41.3 ± 4.2	32.8 ± 1.7	3.49 ± 0.14	4.56 ± 0.13	3.72 ± 0.27	16.39 ± 1.09
Leu-10	45.1 ± 3.5	30.5 ± 2.6	3.35 ± 0.21	4.45 ± 0.11	3.93 ± 0.15	16.38 ± 1.32
Leu-11	50.6 ± 6.4	28.7 ± 3.2	3.24 ± 0.16	4.44 ± 0.23	4.51 ± 0.32	16.70 ± 1.53
^a The unit is g/L; ND: Not detected; ^b The unit is 10 ⁻⁴ nmol/(10 ⁴ cells); ND: Not detected; -: No test.						

Replacement of glyceraldehyde 3-phosphate dehydrogenase to restore NADPH regeneration and L-leucine production

As mentioned above, the strain Leu-1 is an NADPH-auxotrophic *C. glutamicum* strain, thus abolishing cell growth and L-leucine production on glucose (Fig. 2). In 2018, NADPH-auxotrophic *E. coli* strain was used as chassis cells to assess the capacity of different enzymes to biosynthesize NADPH (Lindner et al. 2018). In this study, we tried to use strain Leu-1 as chassis cells to investigate the effect of exogenous NADPH-supplying pathway on L-leucine production. Previous researchers indicated that replacing the native NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (NAD-GapDH) with the nonphosphorylating NADP-GapDH allows cells to produce 2 molecules of NADPH rather than NADH per molecule of glucose (Martinez et al. 2008). Thus, we replaced the native NAD-GapDH with the NADP-GapDH from *C. acetobutylicum* in strain Leu-1, and the resultant strain *C. glutamicum* XL-1 ΔZM_{I_{Cg}::I_{Sm}}G_{Cg}::G_{Ca} (i.e., strain Leu-2) restored the regenerative power of NADPH using glucose as carbon source (Table 2). As a control, we also replaced the NAD-GapDH_{Cg} with NADP-GapDH_{Ca} in strain XL-1, and the resultant strain *C. glutamicum* XL-1 G_{Cg}::G_{Ca} obviously increased the intracellular NADPH level (Table 2). In addition, The maximum specific growth rate (μ_{max}) for the parental strain XL-1 and the GapDH modified strains Leu-2 and XL-1 G_{Cg}::G_{Ca} were 0.35 h⁻¹, 0.32 h⁻¹ and 0.19 h⁻¹, respectively. And the final OD₅₆₂ of these above strains are listed in Table 2. As expected, the strain Leu-2 produced 16.2 ± 1.1 g/L of L-leucine because of the restoration of NADPH supply, which was slight higher than that of parental strain XL-1 (15.3 ± 1.2 g/L of L-leucine)(Fig. 3). It is worth noting that the productivity (i.e., the L-leucine yield per OD₅₆₂) of strain Leu-2 was obvious higher than that of strain XL-1 {0.468 vs 0.387 g/(L•OD₅₆₂); Fig. 3}. In addition, strain XL-1 G_{Cg}::G_{Ca} only produced 14.1 ± 1.8 g/L of L-leucine (Fig. 3), even though the intracellular NADPH level in strain XL-1 G_{Cg}::G_{Ca} was higher than that in strain Leu-2 and strain XL-1 (Table 2). However, the productivity of strain XL-1 G_{Cg}::G_{Ca} was 0.534 g/(L•OD₅₆₂), which was 14.1% and 40.0% higher than that of strain Leu-2 and strain XL-1, respectively (Fig. 3).

Enhancing the carbon flux in L-leucine biosynthetic pathway to further promoting L-leucine production

There are seven enzyme-catalyzed reactions from pyruvate in L-leucine biosynthetic pathway (Fig. 1). In order to enhance the carbon flux in L-leucine biosynthetic pathway, we tried to over-express the genes *ilvBN* (encoding acetohydroxyacid synthase, AHAS, represented by "BN"), *ilvC* (encoding AHAIR, represented by "C"), *leuA* (encoding α-isopropylmalate synthase, IPMS, represented by "A") and *ilvE* (encoding BCAT, represented by "E") by expression vector

pEC-XK99E. In addition, the mutated AHAIr (encoded by *ilvC^M*, represented by “C^M”), which uses NADH as factor rather than NADPH, was also over-expressed. The resulted plasmids pEC-ABNCE and pEC-ABNC^{ME} was transferred into strain Leu-2, and the cell growth, L-leucine yield as well as the intracellular NADH and NADPH level in the resultant recombinant strains were determined. The resultant strain Leu-2/pEC-ABNCE showed the similar cell growth with the parental strain XL-1, whereas the resultant strain Leu-2/pEC-ABNC^{ME} obviously decreased the cell growth as compared with the parental strain XL-1 (Table 2). In addition, the intracellular NADPH level of strain Leu-2/pEC-ABNCE was decrease to 2.56×10^{-4} nmol/(10⁴ cell), which was 64.3% lower than that of strain Leu-2 (Table 2). However, the strain Leu-2/pEC-ABNC^{ME} exhibited a slight decrease in the intracellular NADPH level as compared with the strain Leu-2 (Table 2). As expected, strain Leu-2/pEC-ABNCE produced 26.3 ± 1.7 g/L of L-leucine in 72-h shake flask fermentation, which was about 1.6 and 1.2 times higher than that of strain Leu-2 (16.2 ± 1.1 g/L) and strain Leu-2/pEC-ABNC^{ME} (22.6 ± 2.3 g/L), respectively (Fig. 3). It is worth noting that strain Leu-2/pEC-ABNC^{ME} showed the highest productivity of L-leucine {0.743 g/(L•OD₅₆₂)}, followed by the strain Leu-2/pEC-ABNCE {0.646 g/(L•OD₅₆₂)} and strain Leu-2 {0.468 g/(L•OD₅₆₂)}.

Analyzing the promoter activity of key enzymes involved in NADPH regeneration to determine the key pathway for NADPH regeneration

As mentioned above (Table 2, Fig. 3), strain Leu-2/pEC-ABNC^{ME} showed the high intracellular NADPH level and L-leucine productivity despite of the low L-leucine yield. In contrast, strain Leu-2/pEC-ABNCE exhibited the low intracellular NADPH level and L-leucine productivity as well as the high L-leucine yield. We speculated that the L-leucine production in strain Leu-2/pEC-ABNCE was limited because of the shortage of NADPH. Thus, future research should focus on improving NADPH supply by optimizing the NADPH biosynthetic pathway. To determine the promoter activity of *zwf*, *gnd*, *icd* and *malE* in *C. glutamicum* XL-1, their upstream regions of these genes were cloned in the promoter-probe vector pDXW-11, giving rise to the constructs pDXW-11-*P_{zwf}*, pDXW-11-*P_{gnd}*, pDXW-11-*P_{icd}* and pDXW-11-*P_{malE}* (Fig. 4a). Based on the analysis results from BDGP (Berkeley Drosophila Genome Project, https://www.fruitfly.org/seq_tools/promoter.html), BPROM (<http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>) and reported by Pátek and Nešvera (Miroslav Patek & Nesvera, 2013), about 400 bp fragment upstream from initiation codon of these genes included all of promoter elements, and the potential -10 and -35 regions as well as the transcriptional start site are summarized in Table 3. The transcriptional activity of these cloned fragments including promoters in *C. glutamicum* was investigated using the *cat* reporter gene (encoding CAT) in the extracts of cells cultivated in LBG medium. The CAT-coding gene in plasmid pDXW-11 was successfully expressed under the control of these mentioned promoters (Fig. 4b). As can be seen from Table 3, in addition, the promoters *P_{zwf}* and *P_{gnd}* were strong { 0.365 ± 0.038 U/(mg protein) and 0.406 ± 0.025 U/(mg protein)}, respectively, whereas the promoter *P_{malE}* was rather weak { 0.010 ± 0.003 U/(mg protein)} and the promoter *P_{icd}* was of intermediate strength { 0.092 ± 0.012 U/(mg protein)}. These results indicated that the PP pathway is the key pathway for NADPH regeneration and the tricarboxylic acid (TCA) cycle comes second, whereas the transhydrogenase-like cycle hardly works in strain XL-1.

Table 3

Activity of promoters assayed by measuring the specific activity of chloramphenicol acetyltransferase (CAT) in cell extracts of *C. glutamicum*

Promoter	The key elements of promoter ^a	-10 region ^b	CAT (U/mg protein)	Upstream from ORF ^c
<i>P_{zwf}</i> ^d	gacatgaaatcgaattagttcgatcttattgtgccc	tcttat	0.365 ± 0.038	42 bp
<i>P_{gnd}</i> ^d	ggatttccacctctgtgacgtgtaaaatcgccacta	taaaat	0.406 ± 0.025	47 bp
<i>P_{malE}</i> ^e	ttttgttgagctacagatttagctagtgtttttgtcca	tgtttt	0.010 ± 0.003	69 bp
<i>P_{icd}</i> ^e	taggcgcagcaattagtagaactgtattctaggtagctg	tattct	0.092 ± 0.012	30 bp
<i>P_{gapA}</i> ^e	tctgctgcgaaatcttgtttccccgctaagtgaggac	taaagt	0.753 ± 0.023	183 bp
<i>P_{zwf}</i> -D5	-	CctAat	0.193 ± 0.021	-
<i>P_{zwf}</i> -D1	-	CcttaA	0.106 ± 0.005	-
<i>P_{zwf}</i> -S6	-	tcttaG	0.035 ± 0.008	-
<i>P_{icd}</i> -T3	-	AatAAat	0.179 ± 0.016	-
<i>P_{icd}</i> -S7	-	tattAt	0.285 ± 0.032	-
<i>P_{icd}</i> -D2	-	tatAAat	0.462 ± 0.027	-
^a The bold and italic letters represent the -35 region, the letters with underline represent -10 region and the bold letters with underline represent the transcriptional start site.				
^b Nucleotide substitutions are capital letters and in bold.				
^c It represents the distance between the transcriptional start site and the initiation codon.				
^d The key elements of promoter were deduced from programs BDGP and BPROM.				
^e The key elements of promoter were referred to the previous results reported by Pátek and Nešvera (Miroslav Patek & Nesvera, 2013).				

Constructing the mutant promoters of the *zwf* and/or *icd*_{Cg} genes to balance the intracellular NADPH and NADH level

As the mention above (Fig. 3), although the strain Leu-2/pEC-ABNC^{ME} showed the lower L-leucine yield than that of strain Leu-2/pEC-ABNCE, it showed the higher productivity than that of strain Leu-2/pEC-ABNCE. These results implied that the strain Leu-2/pEC-ABNC^{ME} accumulates excess intracellular NADPH level but the strain Leu-2/pEC-ABNCE lacks adequate NADPH for L-leucine production. It should be noted that the promoter of *gapA* gene (i.e., P_{gapA}) is a strong promoter because the strain with pDXW-11- P_{gapA} showed the high CAT activity (0.753 ± 0.023 U/(mg protein))(Table 3). In order to efficiently supply NADPH for L-leucine production, the promoters of the *zwf* and/or *icd_{Cg}* genes (i.e., P_{zwf} and P_{icd}) were mutated to regulate the expression level of genes controlled by these promoters. In this study, the -10 region of P_{zwf} and P_{icd} was selected as modified object to regulate promoter activity, and the promoter activity was analyzed the CAT activity using the promoter-probe vector pDXW-11. As can be seen from Table 3 and Figure S2, the mutated promoters with base substitution in conservative positions of -10 regions showed the low activity, whereas base substitution in nonconservative positions showed the positive effects on increasing promoter activity. For optimizing promoter of gene *zwf*, the mutated promoters P_{zwf} -D5, P_{zwf} -D1 and P_{zwf} -S6 (with about 50%, 30% and 10% of activity of native-type P_{zwf} , respectively) were selected for further investigating. In addition, the mutated promoters P_{icd} -T3, P_{icd} -S7 and P_{icd} -D2 (with about 2.0-, 3.0- and 5.0-fold stronger than native-type P_{icd} , respectively) were selected for optimizing promoter of gene *icd*.

Following, the mutated P_{zwf} were introduced into strain Leu-2 to control the expression of *zwf* gene, and the mutated P_{icd} replaced the native promoter of *icd_{Cg}* gene in strain Leu-2 to control the expression of *icd_{Sm}* gene. Subsequently, the overexpression plasmid pEC-ABNCE was transferred into these recombinant strains. The resultant strains with the mutated P_{zwf} (i.e., *C. glutamicum* Leu-2 P_{zwf} -D5/pEC-ABNCE (strain Leu-3), *C. glutamicum* Leu-2 P_{zwf} -D1/pEC-ABNCE (strain Leu-4) and *C. glutamicum* Leu-2 P_{zwf} -S6/pEC-ABNCE (strain Leu-5)) showed the increased yield and productive of L-leucine as compared with the strain Leu-2 (Fig. 5a). Among them, the strain Leu-4 showed the best fermentation performance, which produced 28.6 ± 2.4 g/L of L-leucine with a productivity of 0.769 g/(L•OD₅₆₂) in 72-h shake flask fermentation. In addition, the strains with the mutated P_{icd} (i.e., *C. glutamicum* Leu-2 P_{icd} -T3/pEC-ABNCE (strain Leu-6), *C. glutamicum* Leu-2 P_{icd} -S7/pEC-ABNCE (strain Leu-7) and *C. glutamicum* Leu-2 P_{icd} -D2/pEC-ABNCE (strain Leu-8)) exhibited a 5–20% decrease in L-leucine production and a 10–30% increase in cell growth (Table 2). However, replacement of the native P_{icd} by the P_{icd} -T3 in the strain Leu-4 showed the best L-leucine yield and cell growth (Fig. 5a). The resultant strain *C. glutamicum* Leu-2 P_{zwf} -D1 P_{icd} -T3/pEC-ABNCE (i.e., strain Leu-9) produced 32.8 ± 1.7 g/L of L-leucine and its OD₅₆₂ reached to 41.3 ± 4.2 during cultivated in shake flask for 72 h. In addition, the strain Leu-9 has been a slight increase in the intracellular NADH level and the ratio of NADH/NAD⁺ (Table 2). However, introduction of the strong promoters P_{icd} -S7 and P_{icd} -D2 to control *icd_{Sm}* gene expression (i.e., strain Leu-10 and Leu-11) was harmful to L-leucine production despite increasing the cell growth and the intracellular NADH level (Fig. 5b, Table 2). Therefore, we speculated that the strain Leu-9 with P_{zwf} -D1 and P_{icd} -T3 to respectively control the expression of *zwf* and *icd_{Sm}* exhibited the proper intracellular NADPH and NADH level, and thus beneficial to L-leucine production.

Discussion

For the first time, we report an NADPH-auxotrophic *C. glutamicum* recombinant strain and use it to construct L-leucine high-yielding strain. To do this, an L-leucine-producing strain *C. glutamicum* XL-1 was consecutively modified to give strain XL-1 with high-efficiency of L-leucine production, for example, deletion of the native promoter of the NADPH-regenerating enzymes-coding genes to construct a *C. glutamicum* NADPH-auxotroph strain, introduction of the nonphosphorylating NADP-GapDH to restore NADPH regeneration and optimization of the promoters of the *zwf* and/or *icd_{Cg}* genes to balance the intracellular NADPH and NADH level. As a result, a L-leucine high-producing strain *C. glutamicum* Leu-9 was obtained, which produced 32.8 ± 1.7 g/L of L-leucine with a productivity of 0.794 g/(L•OD₅₆₂) in 72-h shake flask fermentation. These results are encouraging, as far as we know (Table 4), indicating that strain Leu-9 is a competitive platform strain for L-leucine production. These results also inferred that optimization of the biosynthetic pathway of NADPH from an NADPH-auxotrophic strain is easy to reasonably supply NADPH, thus increasing the production of NADPH-dependent valuable chemicals.

Table 4
Overview of the production of L-leucine by metabolic engineered *C. glutamicum*

<i>C. glutamicum</i> strains	Fermentation mode	Fermentation time	Final titers (g/L)	Productivity (g/(L•OD ₅₆₂))	Reference
Leu-9	Shake-flask	72 h	32.8	0.794	This work
CP	Ultrasound-assisted fed-batch	44 h	52.89	0.425 ^a	(Zhang et al., 2021a)
CP	Fed-batch	44 h	53.0	0.504 ^a	(Zhang et al., 2021b)
WL-14	Shake-flask	72 h	28.47	0.811 ^a	(Wang et al., 2020)
Leu14	Shake-flask	72 h	14.05	0.502 ^a	(Luo et al., 2021)
MV-LeuF2	Fed-batch	56 h	23.7	- ^b	(Vogt et al., 2014)
^a Estimated from reference. The relation between DCW and OD562 was referred to the formula: DCW (g/L) = 0.57 × OD ₅₆₂ + 0.23 reported in previous study (Wang et al., 2020).					
^b No computed data.					

In *C. glutamicum*, there are four enzymes involved in NADPH biosynthesis, i.e., Zwf, Gnd, NADP-Icd and MalE (Fig. 1) (Hoffmann et al. 2021; Xu et al. 2019). It is clear from this study of the roles of NADPH-regenerating enzymes that different NADPH-regenerating enzymes show a huge difference in catalyzing the NADPH regeneration in *C. glutamicum* (Table 2 and Fig. 2). As previous reports (Lindner et al. 2018), our results show that Zwf and Gnd in PP pathway are the

main enzymes for NADPH regeneration, in which the promoters P_{zwf} and P_{gnd} from strain XL-1 show the strong activity (Table 3). It is worth noting that the native P_{icd} from strain XL-1 show the weak activity (Table 3), which is different from previous reports (Aich et al. 2001; Han et al. 2008). Han et al. pointed out that the transcript level of *icd* from *C. glutamicum* R is high (i.e., 10 ~ 25) in the presence of most of carbon sources, except citrate, succinate and malate (Han et al. 2008). In addition, Aich et al. agreed that the *icd* promoter from *E. coli* is a strong promoter (Aich et al., 2001). Previous studies have already indicated that the activity of *icd* promoter is regulated by secondary substances and accessory proteins (Prost et al. 1999; Shechter et al. 2003). Moreover, base mutation in promoter including -10 region, -35 region and upstream of -35 region will lead to change the promoter activity (Holatko et al. 2009; Vasicova et al. 1999). Given that the strain XL-1 is mutant strain engineered by repeated random mutagenesis (Wang et al. 2019b), random genetic mutation may be happen in some structure genes and/or *icd* promoter and furthermore changes the amount of secondary substances and accessory proteins as well as promoter activity. As expected, inactivation of these NADPH-regenerating enzymes in *C. glutamicum* leads to no NADPH biosynthesis in the resultant strain Leu-1 except using gluconate as carbon source (Table 2 and Fig. 2). The similar results are also found in previous research, in which the NADPH-auxotroph strain *E. coli* NADPhaux grew well only using gluconate as carbon source (Lindner et al. 2018).

Biosynthesis of 1 molecule of L-leucine needs 2 molecules of NADPH (Wang et al. 2019b). To meet the requirement of NADPH for L-leucine production, the native NAD-GapDH was replaced by the NADP-GapDH in strain Leu-1 and original strain XL-1. Since NADP-GapDH allows cells to produce 2 molecules of NADPH per molecule of glucose (Martinez et al. 2008), the resultant strain Leu-2 restored the cell growth and L-leucine production using glucose as carbon source (Table 2, Fig. 3). However, the L-leucine productivity of strain XL-1 $G_{Cg}:G_{Ca}$ was higher than that of strain Leu-2 and strain XL-1, even though the L-leucine yield of strain XL-1 $G_{Cg}:G_{Ca}$ was lower than that of strain Leu-2 and strain XL-1 (Table 2, Fig. 3). In addition, the strain Leu-2 showed the lower the cell growth than that of strain XL-1 despite the higher L-leucine production (Table 2). We speculate that the NADPH supply is inadequate to meet the requirement of NADPH for L-leucine production in strain XL-1, whereas strain Leu-2 and strain XL-1 $G_{Cg}:G_{Ca}$ accumulates excess NADPH and thus to limit the cell growth. This speculation has been proved during enhancement of the carbon flux in L-leucine biosynthetic pathway in strain Leu-2. As can be seen from Table 2, strain Leu-2/pEC-ABNCE showed the similar cell growth with the parental strain XL-1 and the higher than that of strain Leu-2. By contrast, the resultant strain Leu-2/pEC-ABNCE^{ME} obviously decreased the cell growth as compared with the parental strain XL-1 (Table 2). The difference between pEC-ABNCE and pEC-ABNCE^{ME} is the AHAIr-coding gene *ilvC* that needs NADPH as cofactor to catalyze the biosynthesis of α,β -dihydroxyisovalerate. However, the mutated AHAIr (encoded by gene *ilvC^M*, abbreviated to *C^M*) uses NAD as cofactor to catalyze the biosynthesis of α,β -dihydroxyisovalerate because of the mutant AHAIr with three amino acid mutations (i.e., S34G, L48E, and R49F) (Hasegawa et al. 2013). As a result, the intracellular NADPH level of strain Leu-2/pEC-ABNCE^{ME} was slightly decreased while that of strain Leu-2/pEC-ABNCE was significantly decreased (Table 2). Although strain Leu-2/pEC-ABNCE produced more L-leucine titer than strain Leu-2/pEC-ABNCE^{ME} (26.3 ± 1.7 g/L v.s. 22.6 ± 2.3 g/L), but the productivity of L-leucine was lower than that of strain Leu-2/pEC-ABNCE^{ME} (0.646 g/(L•OD₅₆₂) v.s. 0.743 g/(L•OD₅₆₂)). The results demonstrated that the NADPH supply in strain Leu-2/pEC-ABNCE is still limiting factor for L-leucine production. In order to increase NADPH supply in strain Leu-2/pEC-ABNCE, the key enzymes in NADPH regeneration (i.e., *Zwf* and *Icd*) were reasonably modified again. To do this, the -10 region in promoters of the *zwf* and/or *icd_{Cg}* genes (i.e., P_{zwf} and P_{icd}) were mutated to regulate the expression level of *zwf* and *icd_{Cg}*. Consistent with the previous results (Holatko et al. 2009; Vasicova et al. 1999), the mutated promoters with base substitution in conservative positions of -10 regions showed the low activity, whereas base substitution in nonconservative positions showed the positive effects on increasing promoter activity (Table 3 and Figure S2). To optimize the expression level of *zwf* and *icd_{Cg}* for balance the intracellular NADPH and NADH level, three mutated promoters of P_{zwf} (i.e., P_{zwf} -D5, P_{zwf} -D1 and P_{zwf} -S6) and three mutated promoters of P_{icd} (i.e., P_{icd} -T3, P_{icd} -S7 and P_{icd} -D2) were selected for further investigating the effect on L-leucine production. As can be seen from Table 2, the intracellular NADPH level was decreased with the decrease of promoter activity of P_{zwf} , whereas the highest L-leucine yield (i.e., 28.6 ± 2.4 g/L) was found in strain Leu-4 with P_{zwf} -D1. These results indicated that excess NADPH supply is bad for L-leucine production. The similar results were also found in previous reports, in which excess NADPH supply will disturbs glucose uptake and cell growth and thus to inhibit the production of L-lysine or L-threonine (Liu et al. 2019; Xu et al. 2019). It is worth noting that the L-leucine yield was not increased but the cell growth was obviously increased during increasing the promoter activity of P_{icd} (Table 2). The similar results were also found in the next study that the mutated P_{icd} with different activity were introduced in the strain Leu-4 (Table 2 and Fig. 5a). One of the reasons is that more NADH was regenerated during increase of the activity of *Icd_{Sm}* (Table 2 and Fig. 5b), and thus to meet the demand of cell growth for energy (Yumnam et al., 2021). It should be noted that the strains with high *Icd_{Sm}* activity showed the low yield and productivity of L-leucine because more carbon source was used for cell growth.

In conclusion, an NADPH-auxotrophic *C. glutamicum* recombinant strain can be constructed by inactivation of *Zwf* and *MalE* as well as replacement of *icd_{Cg}* by *icd_{Sm}* in *C. glutamicum*. The NADPH-auxotrophic *C. glutamicum* strain Leu-1 can be used as chassis cells for constructing L-leucine high-yielding strain based on optimizing the biosynthetic pathway of NADPH. After a series of genetic modifications in strain Leu-1, an L-leucine high-yielding strain Leu-9 was obtained, which produced 32.8 ± 1.7 g/L of L-leucine with a productivity of 0.794 g/(L•OD₅₆₂) in 72-h shake flask fermentation.

Abbreviations

Zwf, glucose-6-phosphate dehydrogenase; *MalE*, and malic enzyme; *Icd*, isocitrate dehydrogenase; GapDH, glyceraldehyde 3-phosphate dehydrogenase; AHAIr, acetohydroxyacid isomeroeductase; BCAT, branched-chain amino acid transaminase; *Gnd*, 6-phosphogluconate dehydrogenase; CAT, chloramphenicol acetyltransferase; ROS, reactive oxygen species.

Declarations

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Data availability

Please contact author for data requests.

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Contributions

Sheng-Ling Chen designed the research. Sheng-Ling Chen and Ting-Shan Liu performed the experiments, analyzed the data, and prepared the figures. Sheng-Ling Chen, Ting-Shan Liu, and Jian-Zhong Xu wrote the manuscript.

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Ethics declarations

Conflicts of interest

There are no conflicts to declare.

Supporting Information

The oligonucleotides used in this study; Strategy used for the construction of the integrative plasmids used for promoter deletion and gene replacement; Effects of mutations in the -10 region of P_{zwf} (a) and P_{lcd} (b) on promoter activity.

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Figures

Figure 1

Schematic representation of the NADPH auxotrophic *C. glutamicum* and modified strategies. NADPH and NADH metabolic pathway are shown in blue arrows and brown arrows, respectively. The red arrows represent the extrinsic routes. The symbols and represent the deletion and the replacement, respectively. Abbreviations: G6P, glucose-6-phosphate; 6PGL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; 3-PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Ace-CoA, acetyl-CoA; ICit, isocitrate; α-KG, α-ketoglutarate; Suc, succinate; Mal, malate; OAA, oxaloacetate; 2-AL, α-acetolactate; 2,3-DHIV, α,β-dihydroxyisovalerate; 2-KIV, α-ketoisovalerate; 2-IPM, α-isopropylmate; 2-KIC, α-ketoisocaproate.

Figure 2

Growth phenotypes of recombinant and original strains under the different carbon sources. Figures from “a” to “h” represent strain XL-1, XL-1 ΔZ , XL-1 ΔM , XL-1 $\Delta I_{CG}::I_{SM}$, XL-1 ΔI_{CG} , XL-1 $\Delta Z \Delta M$, XL-1 $\Delta Z \Delta I_{CG}::I_{SM}$ and Leu-1, respectively. The data represent mean values and standard deviations obtained from three independent cultivations.

Figure 3

The synthesis capacity of L-leucine of original strain and recombinant strains in the fermentation medium. The data represent mean values and standard deviations obtained from three independent cultivations.

Figure 4

The promoter activity of key enzymes involved in NADPH regeneration. (a) The strategies for constructing the promoter-probe vectors. (b) Analyzing the CAT expression of strains under the different promoters by SDS-PAGE. Lane 1 *E. coli* pDXW-11; Lane 2 *E. coli* pDXW-11-*P_{gapA}*; Lane 3 *E. coli* pDXW-11-*P_{malE}*; Lane 4 *E. coli* pDXW-11-*P_{icd}*; Lane 5 *E. coli* pDXW-11-*P_{zwf}*; Lane 6 *E. coli* pDXW-11-*P_{gnd}*; Lane M Protein marker. The blot in the red box is the CAT.

Figure 5

The positive effects of the mutate promoters of the *zwf* and *icd_{Cg}* on L-leucine production. (a) L-leucine yield and productivity of recombinant strains with different promoters of the *zwf* and *icd_{Cg}*. (b) NADH level and NADH/NADPH ratio of recombinant strains with different promoters of the *zwf* and *icd_{Cg}*. The data represent mean values and standard deviations obtained from three independent cultivations.

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