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Electronic Supplementary Information

Redesign and engineering of a dioxygenase targeting biocatalytic synthesis of 5-hydroxyl leucine

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1. General materials

All standard reaction reagents or materials were purchased from Sigma-Aldrich, unless otherwise stated. Synthetic oligonucleotides and primers were obtained from Genewiz (Beijing, China). All primers used for PCR are listed in Table S1.

2. Expression and enzymatic properties of NpLDO

2.1 Protein expression and purification

The recombinant pET22b(+) plasmid harboring the wild-type NpLDO gene (GenBank No. RCJ32143.1) was transformed into *Escherichia coli* (*E. coli*) BL21(DE3) cells, and cells were grown at 37°C in lysogeny broth (LB) medium containing 40 μg/mL kanamycin. Cells were grown to an optical density (OD_{600}) of 0.8, after which, protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Subsequently, the

culture was incubated at 16°C for 16–18 h. Harvested cells were resuspended in buffer (20 mM Tris-HCl [pH 8.0], 20 mM imidazole, 0.3 M NaCl) and disrupted by sonication. The cell debris were removed by centrifugation at 40,000 × *g* and 4°C for 30 min. The His_6-tagged NpLDO was trapped on Ni-NTA Superflow resin (Qiagen, Hilden, Germany). After washing the resin with wash buffer (20 mM Tris-HCl, pH 8.0, 40 mM imidazole, 0.5 M NaCl, 1 mM DTT), NpLDO was eluted with 5 mL of the elution buffer, which was the same as the wash buffer except 400 mM imidazole was used. The resulting solution containing purified NpLDO was dialyzed against HEPES buffer for the activity assays. The protein concentration was determined by the BCA method.[1,2]

2.2 Characterization of NpLDO

The optimal pH of the purified NpLDO was determined by measuring the activity of the enzyme in 20 mM sodium acetate-acetic acid buffer (pH 4.5–5.5), 20 mM MES buffer (pH 5.5–6.5), 20 mM PBS buffer (pH 6.0–8.0), 20 mM HEPES buffer (pH 7.0–8.0) and 25 mM Tris-HCl buffer (pH 7.0–9.0) at 25°C for 5 min. The optimal temperature was determined by incubating NpLDO at different temperatures (15–65°C). Protein thermostability was determined by measuring the residual activity after incubation of the enzyme at various temperatures from 20 to 65°C for 30 min. The effect of pH on NpLDO stability was tested by incubating purified NpLDO at different pH values (pH 5.0–9.0) for 1 h at 4°C. The activity was then measured under standard reaction conditions.

3. Molecular biology manipulations

3.1 Strains and culture conditions

All primers used for PCR in this study are listed in Table S1, and the used or constructed strains and plasmids are shown in Table S2. *E. coli* DH5α cells, which were cultured in LB medium, were used for vector construction.[3-4] *E. coli* MG1655 cells were used for protein overexpression in LB at 37°C in a shaker flask at 200 rpm. The recombinant *E. coli* strains (*ΔaceAΔsucA*) were routinely precultured in LB

complex M9-salts medium with glycerol and leucine.[5] Kanamycin and/or ampicillin was added to the medium at appropriate concentrations when necessary.

3.2 Engineering of recombinant strains

The CRISPR/Cas9 system was used to delete *aceA* and *sucA* in *E. coli* MG1655 (DE3) cells.[6]

3.2.1 Construction of gRNA plasmids and donor DNA

The gRNA sequence was obtained using the CRISPR RGEN Tools (http://www.rgenome.net). A pair of single stranded DNA sequences was constructed for the target genes, containing the gRNA spacer specific sequence homologous to the pGRB backbone.[7] This annealed pair of single-stranded DNA was ligated into the linearized pGRB plasmid using the NEBuilder HIFI DNA Assembly method, resulting in the gRNA plasmid.

The donor DNA fragment comprising the up and downstream homologous arms for integration was amplified from the *E. coli* genome and fused by overlap-extension PCR. Subsequently, the fused fragment was purified from an agarose gel and used for electroporation.

3.2.2 DNA fragment deletion

To delete the target gene, the plasmid pREDCas9 was introduced into *E. coli* MG1655 competent cells and the cells were made electrocompetent.[8] Thereafter, the prepared gRNA plasmid (100 ng) and donor DNA (200 ng) were electroporated into the electrocompetent cells containing the pREDCas9 plasmid, followed by the immediate addition of 1 mL LB and recovery at 32°C for 2 h. The recovered cells were plated onto LB agar supplemented with ampicillin and spectinomycin, and cultured overnight at 32°C. The verified positive transformants were confirmed by DNA sequencing. The correct colonies were cultivated overnight at 32°C in LB with 0.2% L-arabinose to cure the gRNA plasmid. The pREDCas9 plasmid was cured by cultivation in LB at 42°C for 8 h. The resulting fully cured strain was used for further engineering. Thus, the *ΔaceA* strain and the *ΔaceA ΔsucA* strains were obtained (Table S2).

4. Quantitative RT-PCR analysis

Cells from the mid-log-phase were harvested for total mRNA isolation using the Ultrapure RNA Kit (CWbiotech, Beijing, China), and the transcription levels of the *LGOX*, *CAT* and *NpLDO-WT* genes were obtained via RT-PCR, according to the manufacturer's instructions. The relevant gene transcription levels were calculated using the 2-ΔΔCt method[9] and 16s rDNA as the control. The primers for RT-PCR are listed in Table S1.

5. Bioconversions

5.1 Batch fermentation in 500 mL-shake flasks

The recombinant strains 5HL-1, 5HL-2*Δ*, 5HL-2, 5HL-3, 5HL-4, 5HL-5 and 5HL-6 were streaked onto LB agar plates and incubated overnight. Single colonies were selected and transferred to flasks containing 5 mL of LB medium and cultivated overnight at 37°C and 200 rpm. The resulting seed cultures were used to inoculate 500-mL shake flasks containing 100 mL of fermentation medium and incubated at 37°C and 200 rpm until the OD_{600} reached 0.8, at which point IPTG was added to a final concentration of 0.3 mM. The cultures were further cultivated at 30°C and 200 rpm for 50 h to express the heterologous proteins. The fermentation medium was LB with 200 mM glucose, 5 mM $FeSO_4 \cdot 7H_2O$, 1 mM ascorbic acid, 120 mM glycerol, 100 mM L-leucine, 50 mM L-glutamate and the pH was adjusted to 7.0 using 1 M KOH. Samples were collected every 4 h. The OD_{600} values were measured photometrically and the concentration of 5-HLeu was determined by HPLC.

5.2 Batch fermentation in 3 L-shake flasks

The recombinant strain 5HL-6 was used for medium-scale fermentation. Single colonies were inoculated into 50 mL of LB medium and cultivated overnight at 37°C and 200 rpm. The resulting seed cultures were used to inoculate 3 L-shake flasks containing 1 L of fermentation medium and incubated at 37°C and 200 rpm until the OD_{600} reached 0.8–1, at which point protein expression was induced by adding IPTG

to a final concentration of 0.1 mM, followed by further cultivation at 30°C and 200 rpm for 50 h. The fermentation medium contained LB, 300 mM glucose, 30 mM $(NH_4)_2SO_4$, 3 mM $KH_2PO_4 \cdot 3H_2O$, 1.5 mM $MgSO_4 \cdot 7H_2O$, 15 mM $FeSO_4 \cdot 7H_2O$, 3 mM ascorbic acid, 200 mM L-leucine, 80 mM L-glutamate and the pH was adjusted to 7.0 with 1 M KOH. Samples were taken every 2 h. During the batch culture, the pH was maintained at approximately 7.0 by the addition of $NH_3 \cdot H_2O$. The biomass was monitored by measuring the OD_{600}, and the production of 5-HLeu and consumption of L-leucine were measured by HPLC.

6. Statistical analysis

All experiments were performed in triplicate. One-way analysis of variance (ANOVA) was used as a test for statistical significance with a cutoff value of $p = 0.05$.

7. Supplementary tables and figures

Table S1. Primers used in this work.

Primers	Sequence (5'-3')
NpLDO (WT) site-directed mutation	**Primers**
V77A_F	GCGTTCGAGTACAATCGCATCAGCAAAG
V77A_R	GGTGATGCCAATGCGTTCGATCTTCG
V77L_F	TTGTTCGAGTACAATCGCATCAGCAAAG
V77L_R	GGTGATGCCAATGCGTTCGATCTTCG
V77I_F	GTCTTCGAGTACAATCGCATCAGCAAAG
V77I_R	GGTGATGCCAATGCGTTCGATCTTCG
F78W_F	TGGGAGTACAATCGCATCAGCAAAGC
F78W_R	CACGGTGATGCCAATGCGTTCGATC
F78A_F	GCCGAGTACAATCGCATCAGCAAAGC
F78A_R	CACGGTGATGCCAATGCGTTCGATC
I75A_F	GCCACCGTGTTCGAGTACAATCGC
I75A_R	GCCAATGCGTTCGATCTTCGGGTTC
I75V_F	GTCACCGTGTTCGAGTACAATCGC
I75V_R	CCAATGCGTTCGATCTTCGGGTTC
I70G_F	GGCGAACGCATTGGCATCACCG
I70G_R	CTTCGGGTTCACATTCTGGTAGC
S172C_F	TGCTGGAACCTGTACCTGAAATTCAG
S172C_R	CAGCTGATAGATCACGGTGCCGATC
S172T_F	ACGTGGAACCTGTACCTGAAATTCAG
S172T_R	CAGCTGATAGATCACGGTGCCGATC
K202A_F	GCGCTGGACAGCTACGGCTATGG
K202A_R	ATACTGTTCATCGCCCGGCTGCCATT
K202F_F	TTCCTGGACAGCTACGGCTATGG
K202F_R	ATACTGTTCATCGCCCGGCTGCCAT
V77G_F	GGGTTCGAGTACAATCGCATCAGCAAAG
V77G_R	GGTGATGCCAATGCGTTCGATCTTCG
V77C_F	TGCTTCGAGTACAATCGCATCAGCAAAG
V77C_R	GGTGATGCCAATGCGTTCGATCTTCG
V77T_F	ACGTTCGAGTACAATCGCATCAGCAAAG
V77T_R	GGTGATGCCAATGCGTTCGATCTTCG
V77W_F	TGGTTCGAGTACAATCGCATCAGCAAAG
V77W_R	GGTGATGCCAATGCGTTCGATCTTCG
V77F_F	TTCTTCGAGTACAATCGCATCAGCAAAG
V77F_R	GGTGATGCCAATGCGTTCGATCTTCG

V77D_F	GATTTCGAGTACAATCGCATCAGCAAAG
V77D_R	GGTGATGCCAATGCGTTCGATCTTCG
V77E_F	GAGTTCGAGTACAATCGCATCAGCAAAG
V77E_R	GGTGATGCCAATGCGTTCGATCTTCG
V77H_F	CATTTCGAGTACAATCGCATCAGCAAAG
V77H_R	GGTGATGCCAATGCGTTCGATCTTCG
V77K_F	AAGTTCGAGTACAATCGCATCAGCAAAG
V77K_R	GGTGATGCCAATGCGTTCGATCTTCG
V77M_F	ATGTTCGAGTACAATCGCATCAGCAAAG
V77M_R	GGTGATGCCAATGCGTTCGATCTTCG
V77N_F	AATTTCGAGTACAATCGCATCAGCAAAG
V77N_R	GGTGATGCCAATGCGTTCGATCTTC
V77P_F	CCGTTCGAGTACAATCGCATCAGCAAAG
V77P_R	GGTGATGCCAATGCGTTCGATCTTCGA
V77Q_F	CAGTTCGAGTACAATCGCATCAGCAAAG
V77Q_R	GGTGATGCCAATGCGTTCGATCTTCG
V77R_F	AGGTTCGAGTACAATCGCATCAGCAAAG
V77R_R	GGTGATGCCAATGCGTTCGATCTTCG
V77S_F	TCGTTCGAGTACAATCGCATCAGCAAAG
V77S_R	GGTGATGCCAATGCGTTCGATCTTCG
V77Y_F	TATTTCGAGTACAATCGCATCAGCAAAG
V77Y_R	GGTGATGCCAATGCGTTCGATCTTC
Random mutagenesis	**Primers**
R-NpLDO-F	CGCGGATCCATGGCCGCAACCAGCAACC
R-NpLDO-R	CCCAAGCTTTTAGCTCCATAAGATAATTTCGCCG
MT3 site-directed mutation	**Primers**
MT3_V77A_F	GCGTTCGAGTACAATCACATCAGCAAAG
MT3_V77A_R	GGTGATGCCAATGCGTTCGATCTTCG
Genes editing	**Primers**
*aceA*_UP_F	AGGTGCTCAACAAAGTAAAAGCGGA
*aceA*_UP_R	CAGCAACGGTTGTTGTTGCGTGCAGATGCTCCATAGTTATGTGGTG
*aceA*_DN_F	GCAACAACAACCGTTGCTGGCAACAACAACCGTTGCTGACTGTAG
*aceA*_DN_R	TCGCTTCCAGCAGACGCTCTGCC
*sucA*_UP_F	CGCGAAAAACTCGACGGGCT GTATG
*sucA*_UP_R	CTCATTGTGTATCCTTTATC GTGATCCCTTAAGCATCTTTTTTATGC
*sucA*_DN_F	GATGCTTAAGGGATCACGATAAAGGATACACAATGAGTAGCGTAG
*sucA*_DN_R	TCGCTTCCAGCAGACGCTCTGCC
*aceA*_gRNA_F	AGTCCTAGGTATAATACTAGTGATCAGATCCAATGGTCCGCGTTTTAG AGCTAGAA
*aceA*_gRNA_R	TTCTAGCTCTAAAACGCGGACCATTGGATCTGATCACTAGTATTATAC CTAGGACT

*sucA*_gRNA_F	AGTCCTAGGTATAATACTAGTCTGACTCGGTTGACGCTAACGTTTTAG AGCTAGAA
*sucA*_gRNA_R	TTCTAGCTCTAAAACGTTAGCGTCAACCGAGTCAGACTAGTATTATAC CTAGGACT
*gltA*_pCOLA_F	CGCGGATCCATGGCTGATACAAAAGCAAAACTCA
*gltA*_pCOLA_R	CCCAAGCTTTTAACGCTTG ATATCGCTTT TAAAGTC
*icd*_pCOLA_F	GGGAATTCCATATGATGGAAAGTAAAGTAGTTGTTCCGG
*icd*_pCOLA_R	CGGGGTACCTTACATGTTTTCGATGATCGCGTC
LGOX_pCOLA_F	CGCGGATCCATGACCACCGACACAGCACGTCG
LGOX_pCOLA_R	CCCAAGCTTTTAGCTGGTTAATGCTTCTTCGCGC
NpLDO_pCOLA_F	GGGAATTCCATATGATGGCCGCAACCAGCAACCAG
NpLDO_pCOLA_R	CCGCTCGAGTTAGCTCCATAAGATAATTTCGCCG
LGOX_CAT_pCOLA_R	CTCGACCGTTTCGACCTGCTGTAA
CAT_pCOLA_F	ATGAGCACGTCAGACGATATCCATAAC
CAT_pCOLA_R	CCCAAGCTTTTACAGCAGGTCGAAACGGTCGAGG
RBS2_LGOX-F	CTCGACCGTTTCGACCTGCTGTAAAAGCTTTCTAGAAGAACAATAC
RBS2_CAT-R	GTTATGGATATCGTCTGACGTGCTCATGGATATCGTCTGACGTGCT
LGOX_CAT_pCOLA_MT3_F	GGGAATTCCATATGGCCGCAA CCAGCAACCAG
LGOX_CAT_pCOLA_MT3_R	CCGCTCGAGTTAGCTCCATAAGACAATTTCGCCG
RT-PCR	**Primers**
MT3_RT_F	TGGCCGCAACCAGCAACCAGAT
MT3_RT_R	TGTTCATCGCCCGGCTGCCATT
LGOX_RT_F	ACCGGTGCCGAACGTGCAAATG
LGOX_RT_R	TGCGCCGTTGGTCCATGTCTTG
CAT_RT_F	ATGTGAACGCCGCAGCCGTTCG
CAT_RT_R	TGCGTGCGGTGGCGGAAGTTTA
16s_F	TCTTGACATCCACAGAACTT
16s_R	TAACCCAACATTTCACAACA

Table S2 Bacterial strains and plasmids used in this work.

Name	Relevant characteristics	Source or Reference
Strains		
MG1655	Wild-type	This lab
MG1655 (DE3)	Protein expression host	This lab
DH5α	Cloning host	This lab
5HL-WT	MG1655(DE3)	This lab
5HL-1*Δ*	MG1655(DE3) *ΔaceA*	This work
5HL-2*Δ*	MG1655(DE3) *ΔaceA ΔsucA*	This work
5HL-1	5HL-1*Δ*/ pCOLA-1/ pET-Np	This work
5HL-2	5HL-2*Δ*/ pCOLA-1/ pET-Np	This work
5HL-3	5HL-2*Δ*/ pTrc-ig / pET-Np	This work
5HL-4	5HL-2*Δ*/ pTrc-ig/ pCOLA-2	This work
5HL-5	5HL-2*Δ*/ pTrc-ig/ pCOLA-3	This work
5HL-6	5HL-2*Δ*/ pTrc-ig/ pCOLA-4	This work
Plasmids		
pCOLADuet-1	lacI, T7lac, Kan^R	This lab
pTrc99a	pBR322 ori, Trc, Amp^R	This lab
pET22b(+)	Amp^R, *E. coli* expression vector	This lab
pET28a(+)	Kan^R, *E. coli* expression vector	This lab
pQE-80L	Kan^R, *E. coli* expression vector	This lab
pCOLA-1	pCOLADuet-1, *icd*, *gltA*	This work
pCOLA-2	pCOLADuet-1, LGOX, NpLDO	This work
pCOLA-3	pCOLADuet-1, LGOX, CAT, NpLDO	This work
pCOLA-4	pCOLADuet-1, LGOX, CAT, MT3	This work
pTrc-ig	pTrc99a, *icd*, *gltA*	This work
pET-Np	pET-22b(+), NpLDO	This work
pQE-Np	pQE-80L, NpLDO	This work
pET-M1	pET-22b(+), MT1	This work
pET-M2	pET-22b(+), MT2	This work
pET-M3	pET-22b(+), MT3	This work
pET- LGOX	pET28a(+), LGOX	This work
pET- RBS	pET28a(+), rbs2	Wu et al. (2018)[10]
pREDCas9	Spe_r, Cas9 and λ Red recombinase expression vector	Li et al. (2015)[11]
pGRB	Amp_r, gRNA expression vector	Li et al. (2015)[11]

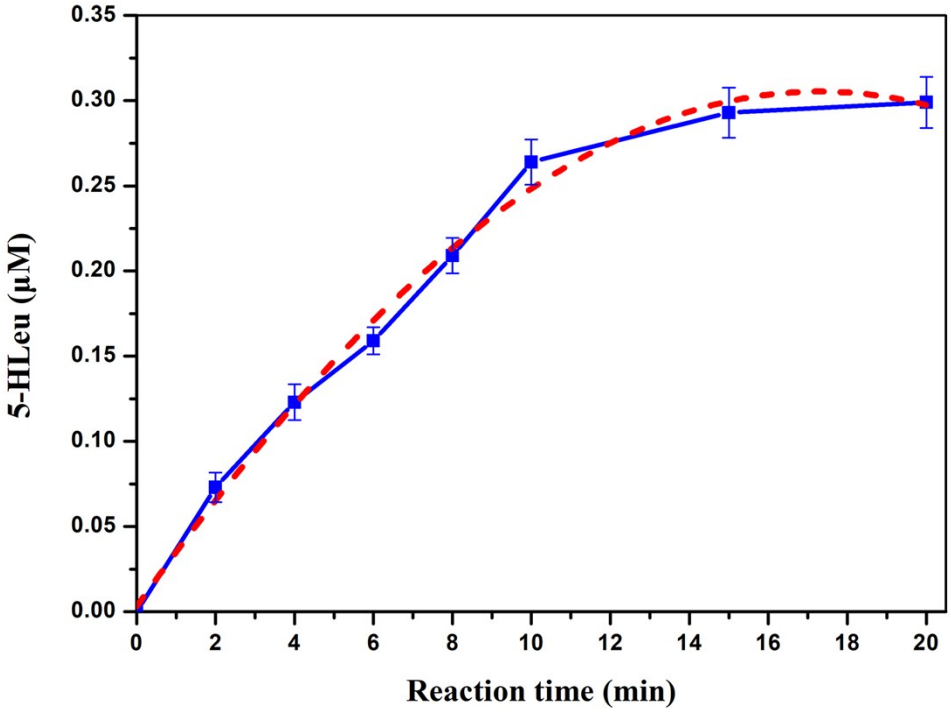

Figure S1. Reaction rate analysis of wild-type NpLDO using leucine as substrates. Reaction conditions: the 100 μL reaction mixture included 10 mM standard substrate, 2 mM α-KG, 0.15 mM $FeSO_4 \cdot 7H_2O$, 2 mM L-ascorbate, 1 mM DTT and 20 mM HEPES buffer (pH 7.0). Error bars are standard deviations (n = 3).

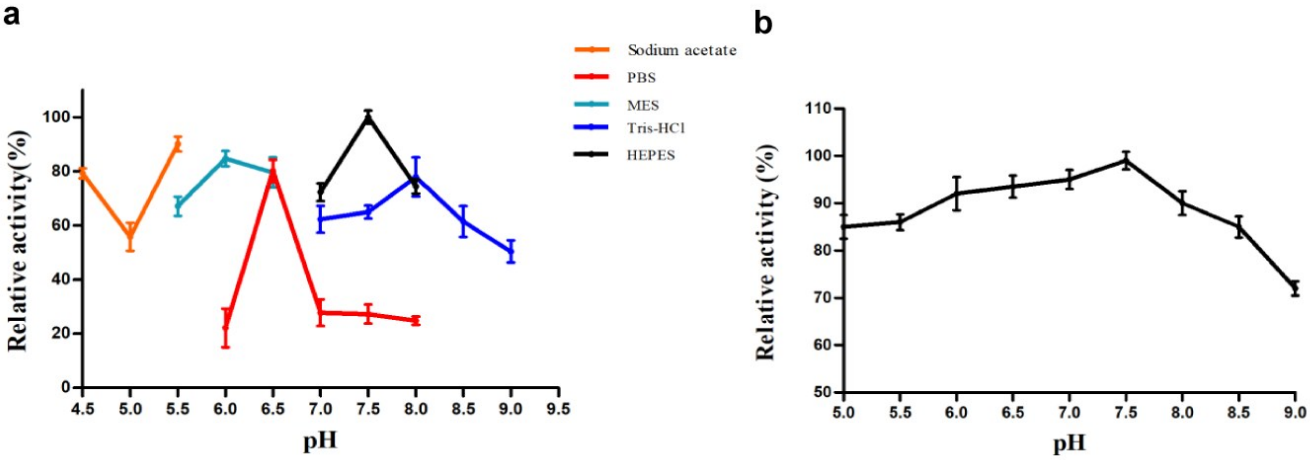

Figure S2. Effect of pH on the activity of NpLDO. (a) pH-dependent activity of NpLDO. Enzyme activity was measured in the reaction mixtures at various pH values by adjusting the pH with 25 mM sodium acetate, PBS, MES, Tris-HCl and HEPES buffers. (b) pH stability of NpLDO. NpLDO was pre-incubated at different pH values for 1 h at 4°C and residual activity was determined under standard assay conditions. Error bars are standard deviations (n = 3).

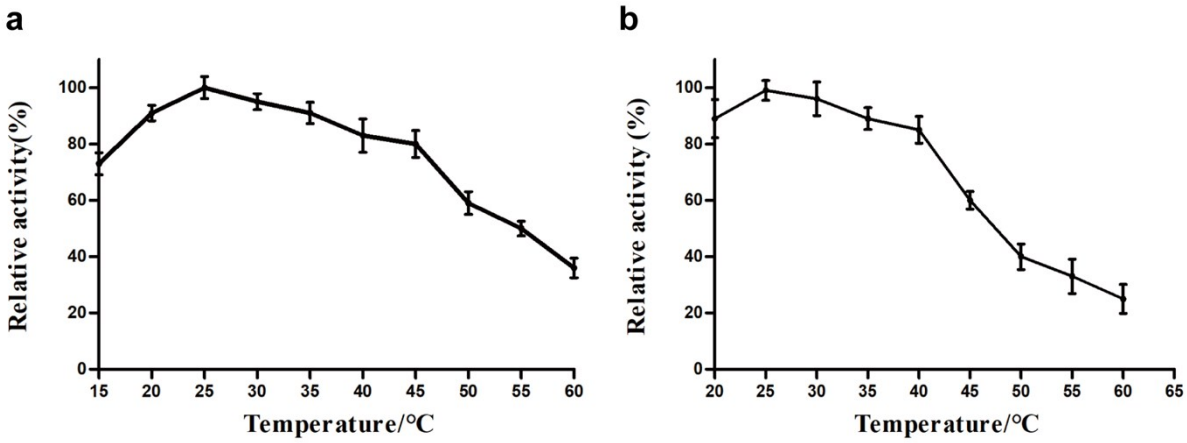

Figure S3. Effect of temperature on the activity of NpLDO. (a) Temperature-dependent activity of NpLDO. Enzyme activity was measured at various temperatures under standard assay conditions. (b) Thermostability analysis of NpLDO. NpLDO was pre-incubated at various temperatures for 30 min and residual activity was determined under standard assay conditions. Error bars are standard deviations (n = 3).

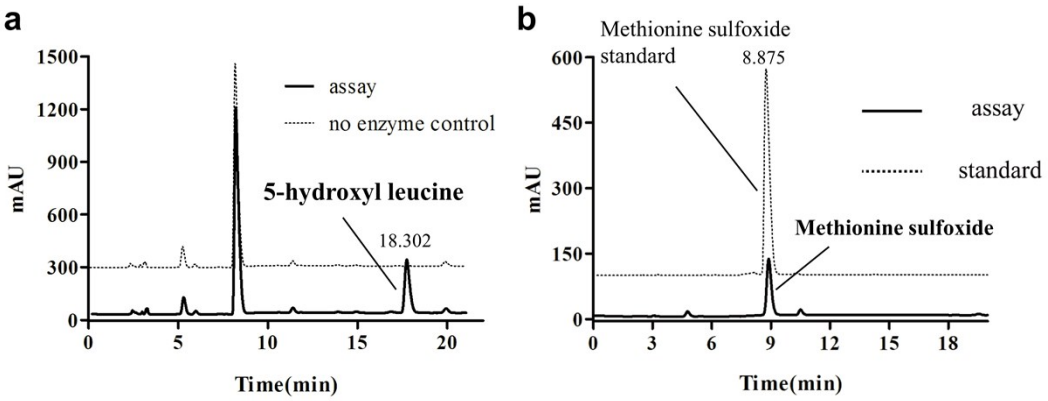

Figure S4. HPLC analysis of (a) 5-hydroxyl leucine and (b) methionine sulfoxide production. Solid lines represent reactions catalyzed by NpLDO, whereas dotted lines represent the reaction mixture in the absence of the enzyme. Peaks in the HPLC chromatogram with retention times of 18.302 min and 8.875 min represent the reaction products.

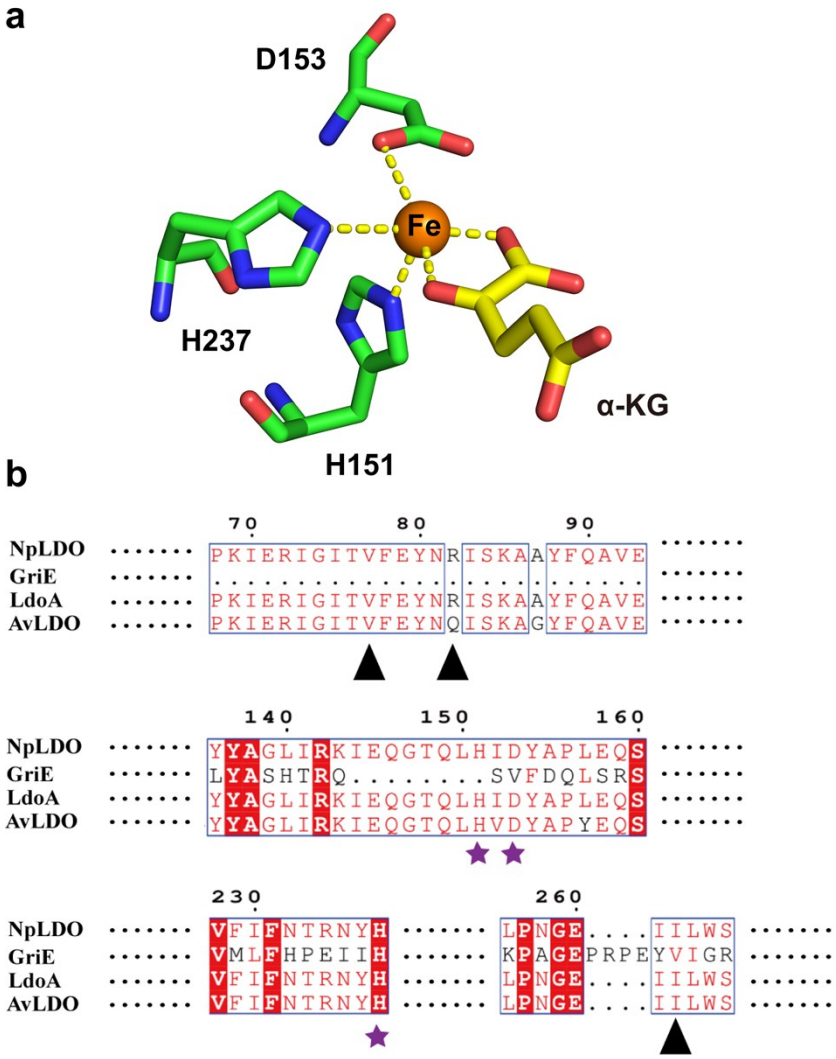

Figure S5. (a) The proposed iron-binding motif. α-KG are shown as yellow sticks, the three activity residues are colored green and the metal is shown as a dark brown sphere. (b) Sequence alignment of NpLDO with L-leucine 5-hydroxylases. NpLDO, L-leucine 5-dioxygenase from *Nostoc punctiforme* NIES-2108; GriE, L-leucine 5-dioxygenase from *Streptomyces* DSM 40835; LdoA, L-leucine 5-dioxygenase from *Nostoc punctiforme* PCC 73102; and AvLDO, L-leucine 5-dioxygenase from *Anabaena variabilis*. Identical amino acids are shaded red. His 151, Asp 153 and His 237 are indicated by five-pointed stars, and the three mutated residues are indicated by triangles.

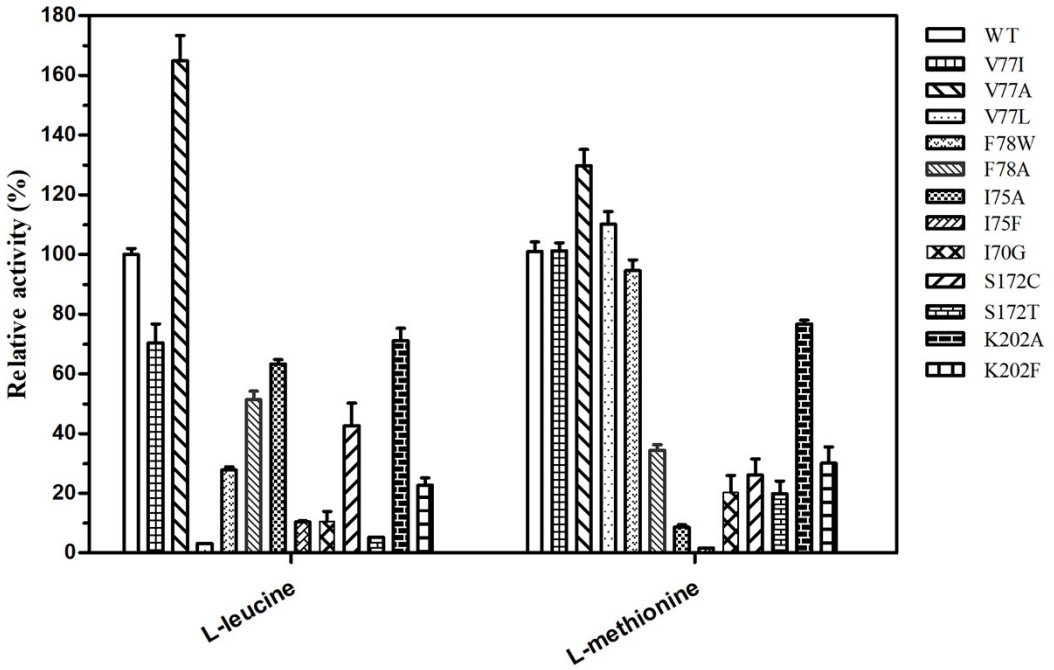

Figure S6. The relative activity of NpLDO mutants toward the two substrates. The activity of wild-type NpLDO toward L-leucine and L-methionine was set at 100 and error bars are standard deviations (n = 3).

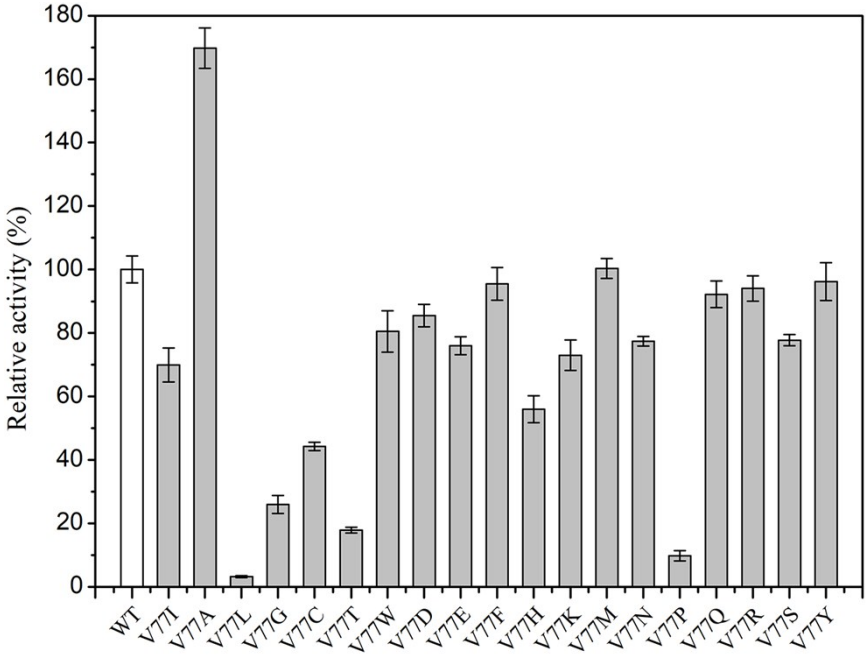

Figure S7. The relative catalytic activity of saturation mutagenesis on V77 toward L-leucine. The enzyme activity of wild-type NpLDO toward L-leucine was set at 100 and error bars are standard deviations (n = 3).

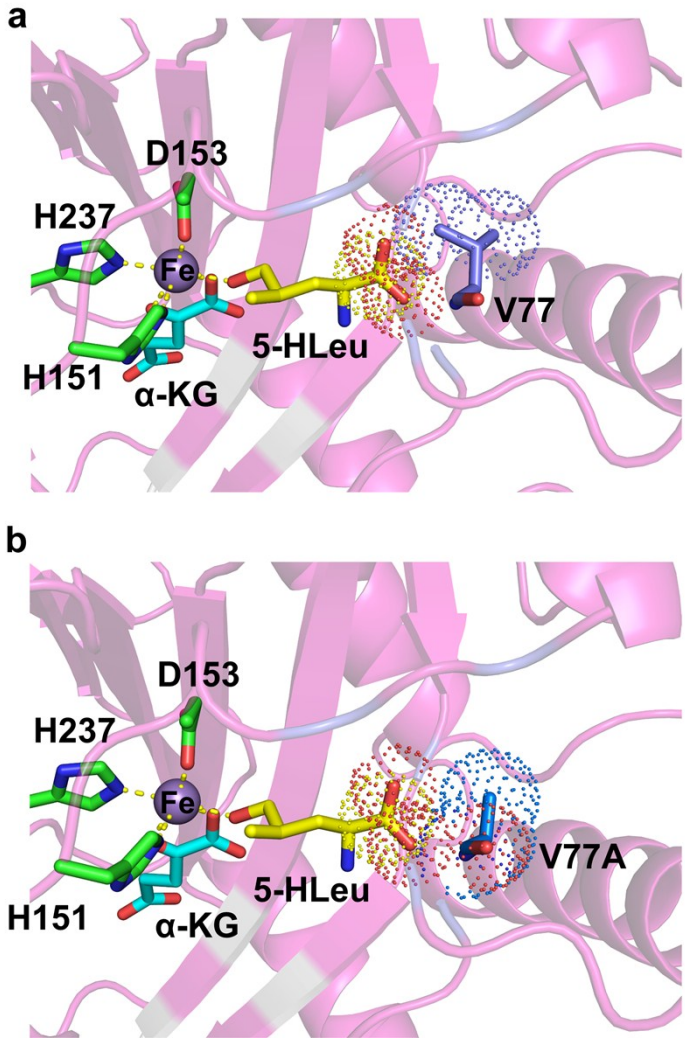

Figure S8. Molecular docking of 5-HLeu to (a) wild-type NpLDO and (b) the V77A mutant. Fe(II) is shown as a deep blue sphere, α-KG is displayed in cyan and 5-HLeu is shown in yellow. Residues V77 and V77A are displayed as purple and deep blue sticks, respectively.

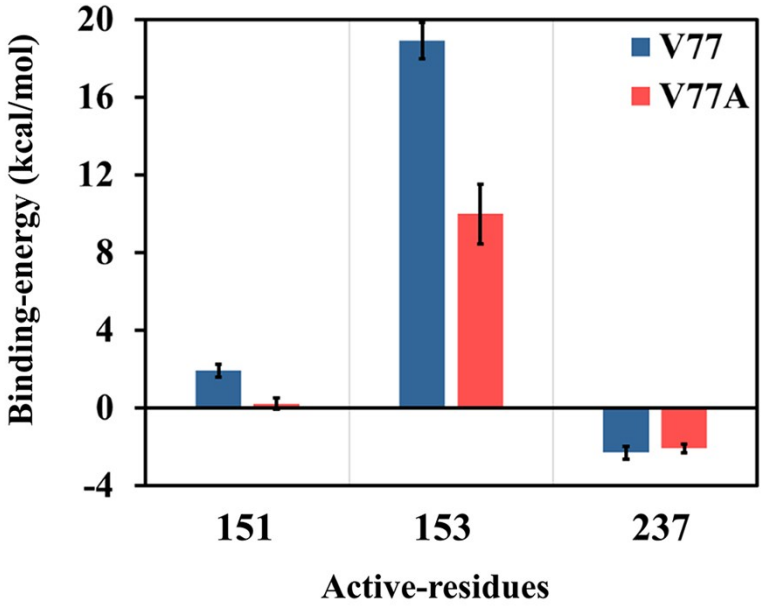

Figure S9. The binding energy between L-leucine and residues around the binding site over 10-ns simulations with respect to their initial positions for the protein in complexes system. The blue and red histograms represent the wild-type protein and the V77A mutant, respectively.

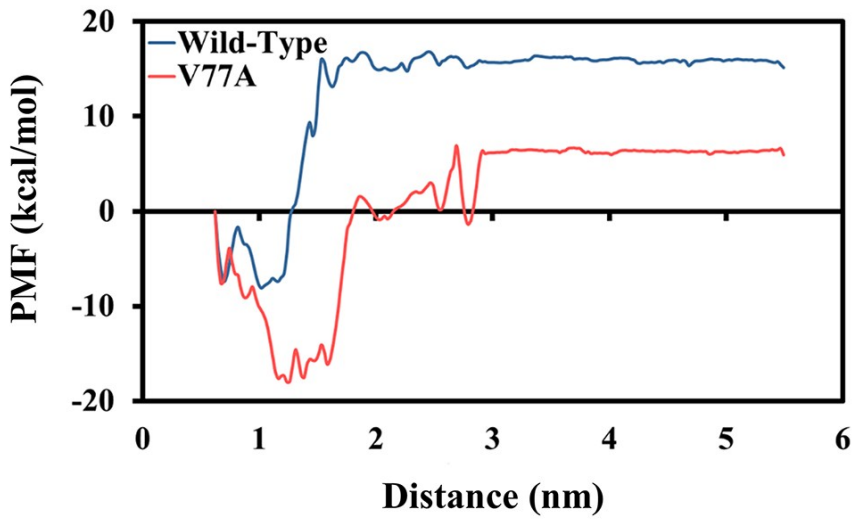

Figure S10. The potential of mean force (PMF) profiles of the wild-type NpLDO and mutants over the distance along the substrate channel.

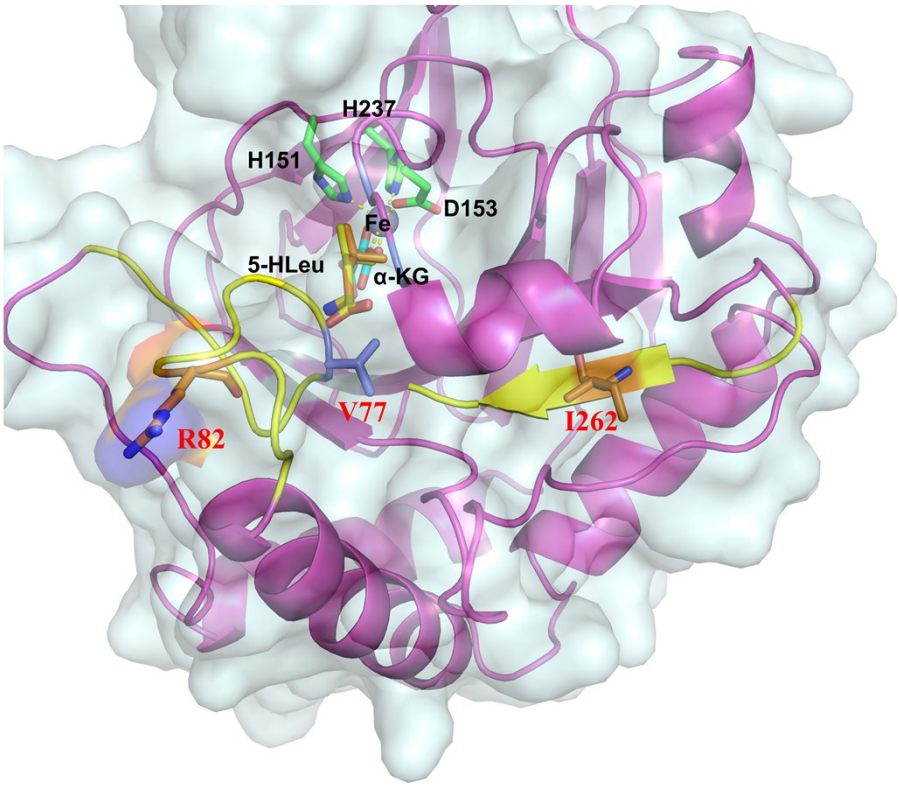

Figure S11. Structural analysis of the NpLDO-substrate complex model. The Q64-K85 loop and G259-L263 β strand are colored pale yellow. Fe(II) is shown as a deep blue sphere, α-KG is displayed in cyan and 5-HLeu is shown in yellow. Residues H151, D153 and H237 are displayed as green sticks, respectively. Residues V77, R82 and I262 are displayed as brown sticks, respectively.

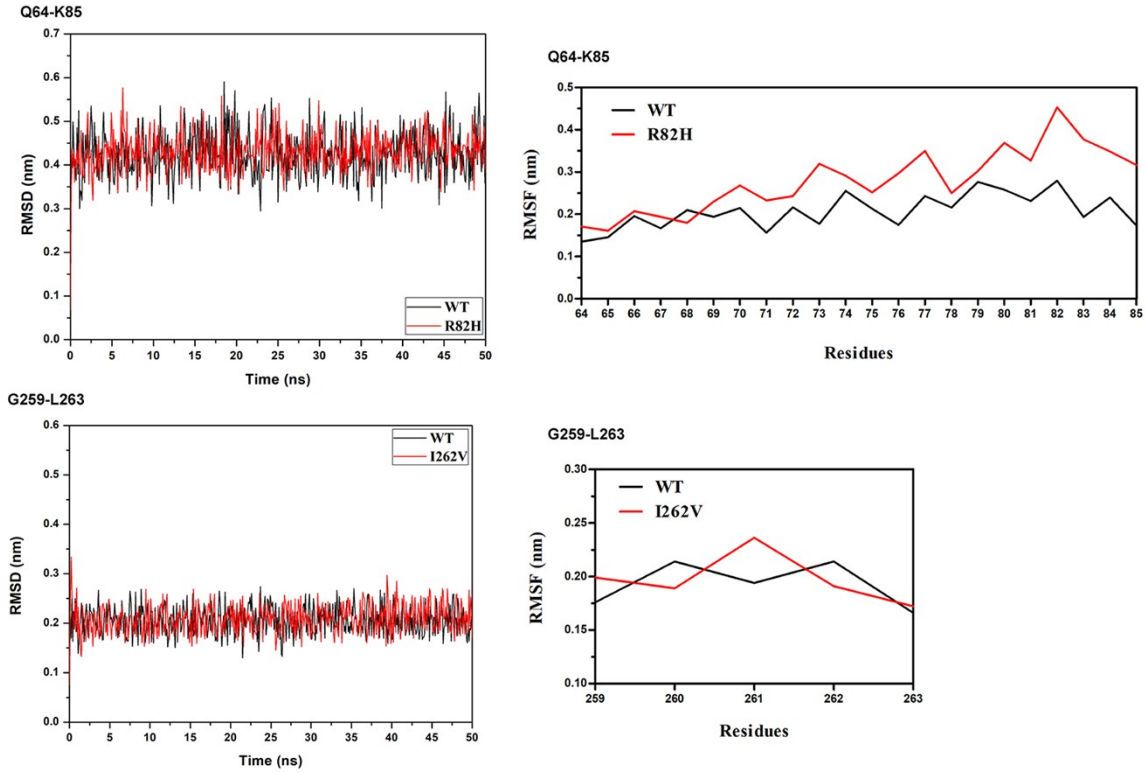

Fig S12. RMSD and RMSF of L-leucine (substrate) and residues over 50-ns MD simulations. Values were calculated by taking the difference from initial positions in the protein complexes. The black and red lines represent the wild-type protein and mutants.

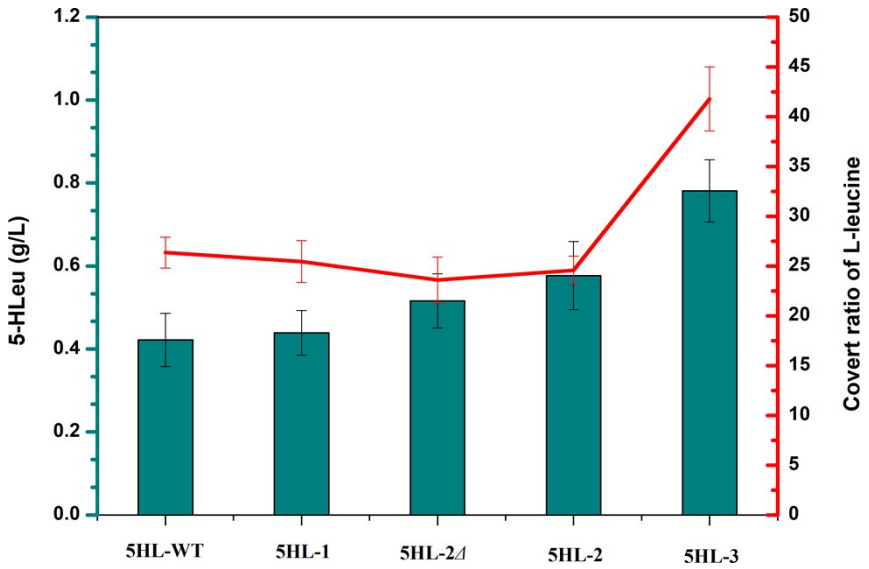

Figure S13. Production of 5-HLeu and the conversion ratio of L-leucine by engineered strains. The column represents the production of 5-HLeu by the engineered strains. The conversion ratio of L-leucine by engineered strains is indicated by the red line and error bars are standard deviations (n = 3).

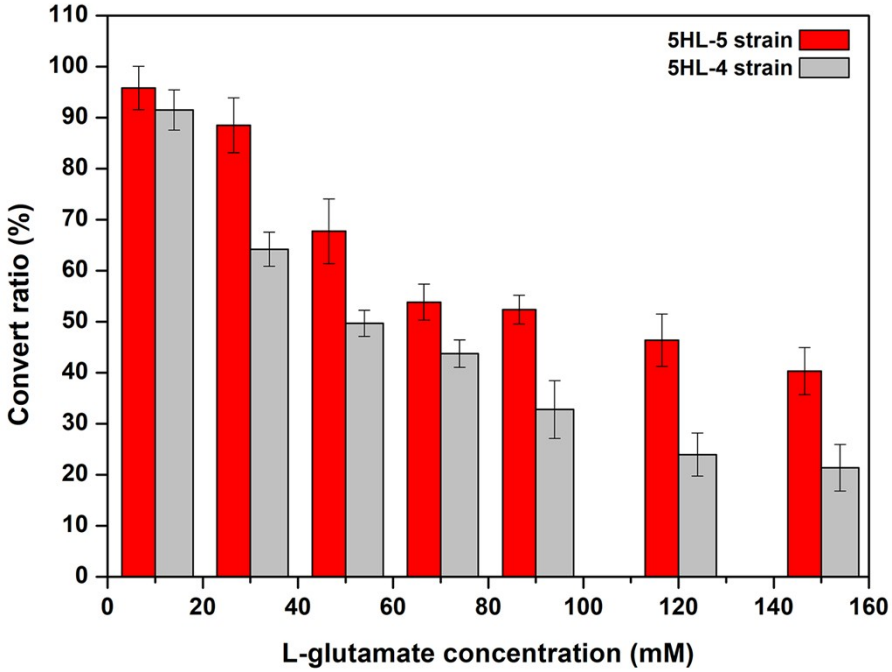

Figure S14. Effect of L-glutamate concentration on the conversion ratio by 5HL-4 and 5HL-5 strains. Error bars are standard deviations (n = 3).

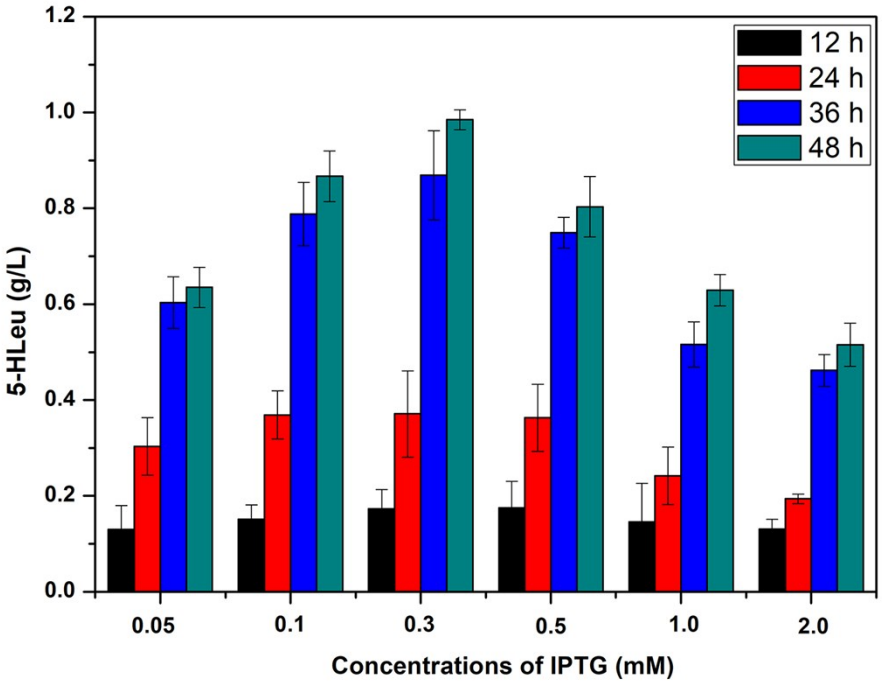

Figure S15. Effect of inducer concentration and induction time (12–48 h) on 5-HLeu production. Error bars are standard deviations (n = 3).

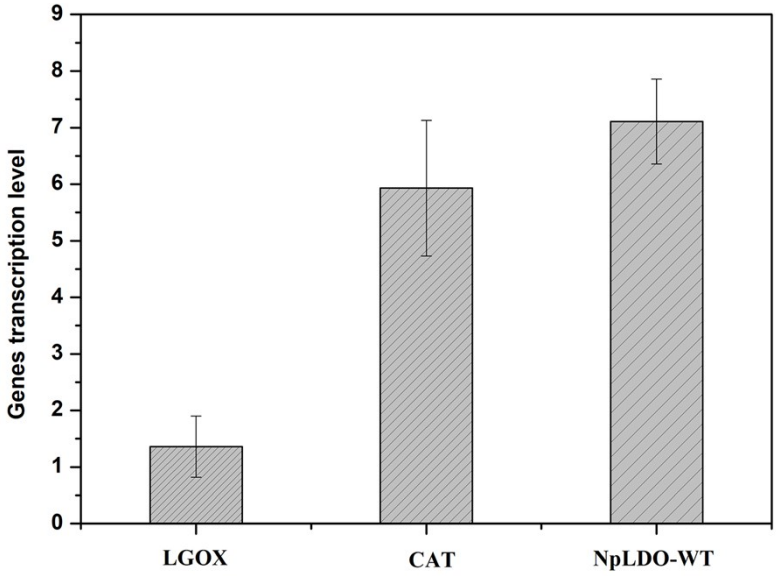

Figure S16. Transcription levels of *LGOX*, *CAT* and *NpLDO-WT* genes during fermentation.

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