One-step biosynthesis of α-keto acids by the L-amino acid deaminase: Biocatalyst construction and process optimization




Guocheng Du

Jiangnan University

Wuxi, China

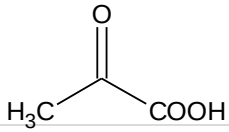
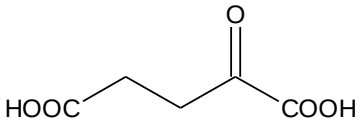
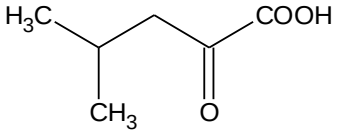
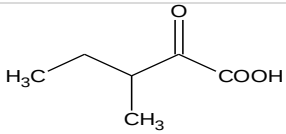
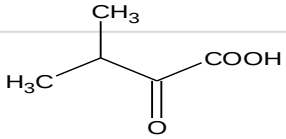
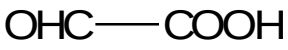
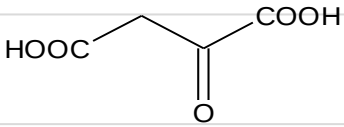
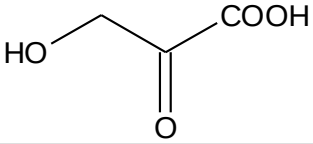
http://www.jiangnan.edu.cn

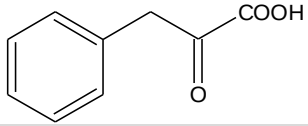
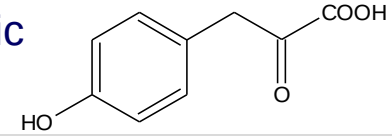
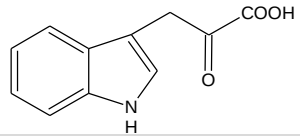
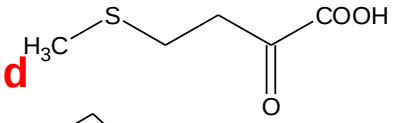
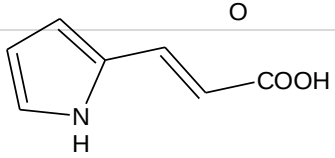
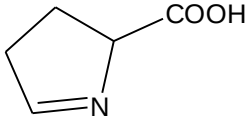
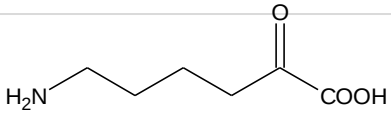
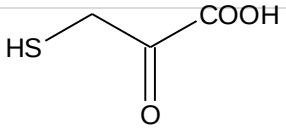
Outline

- 1. Applications of α-keto acids
- 2. Comparison of different production methods
- 3. Biotransformation production of α-keto acids
- 4. Summary

1. Applications of α-keto acids

Types and structures of α-keto acids

Keto acids	Structures
Pyruvic acid	
α-Ketoglutaric acid	
α-Ketoisocaproic acid	
α-Keto-β-methylpentanoic acid	
α-Ketoisovaleric acid	
Glyoxylic acid	
Oxaloacetic acid	
γ-Hydroxy pyruvic acid	

Keto acids	Structures
Phenylpyruvic acid	
4-Hydroxyphenylpyruvic acid	
Indole pyruvic acid	
α-Keto-γ-(methylthio)butyric acid	
Urocanic acid	
Δ'-Pyridinoline-5-carboxylic acid	
α-Keto-e-aminocaproic acid	
γ-Mercaptopyruvic acid	

Applications

α-Keto acids find wide applications in food, medicine, health, and chemical synthesis.

Food

- Substitution for amino acids as food
  Reducing the burden of liver and kidney for patients.

- Improving the flavor and taste
- α-Ketoisocaproic acid can improve the flavor of sausage and cream.

Medicine

- The α-Ketoisocaproic acid can improve immunity by increasing the permeability of lymphocytes.

- The α-Ketoisocaproic acid can improve feeding conversion rate without affecting the quality of the meat.

Applications

Beauty and health

- α-keto acids as additives for skin-care working well on moistening, anti-wrinkle, anti-aging, and anti-anaphylaxis.
- α-keto acids have significant effects on skin whitening and inhibition of black spots.

Chemical synthesis

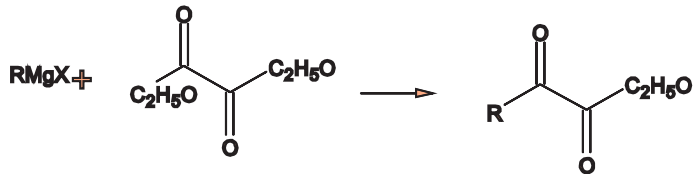
- α-keto acids can be used to synthesize α-hydroxy acids by nucleophilic addition with aldehyde ketone.

2. Comparison of different production methods

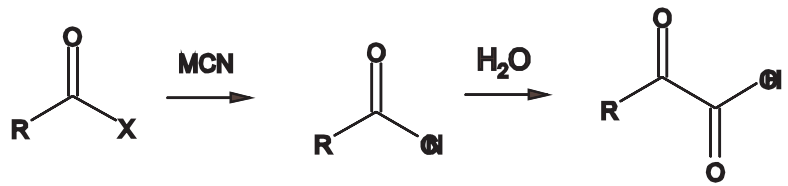
Production methods-Chemical synthesis

Chemical synthesis: the main method to produce keto acids

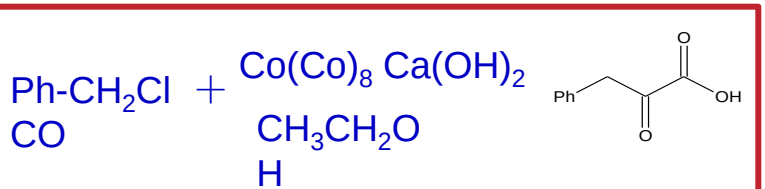
- **Advantages: short reaction path, high conversion rate.**
- **Disadvantages: toxic catalyst, high temperature, pressure, and energy consumption.**



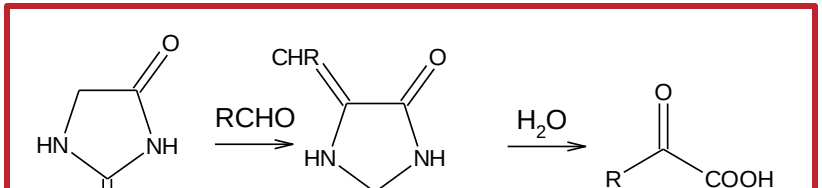
Nucleophilic substitution of grignard reagent and diethyl oxalate



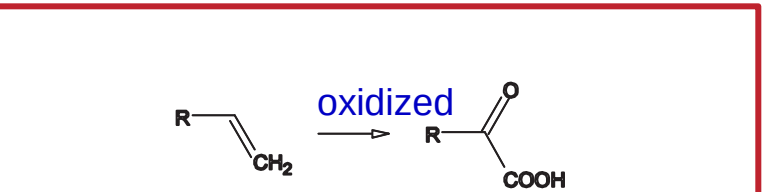
Hydrolysis



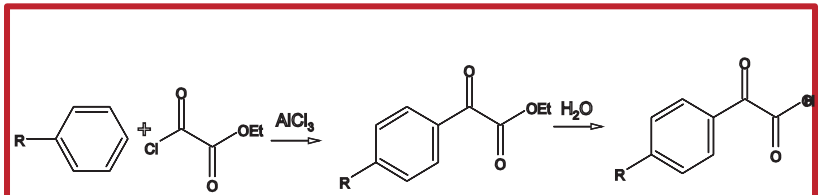
Double carboxylated



Hydantion



Oxidation

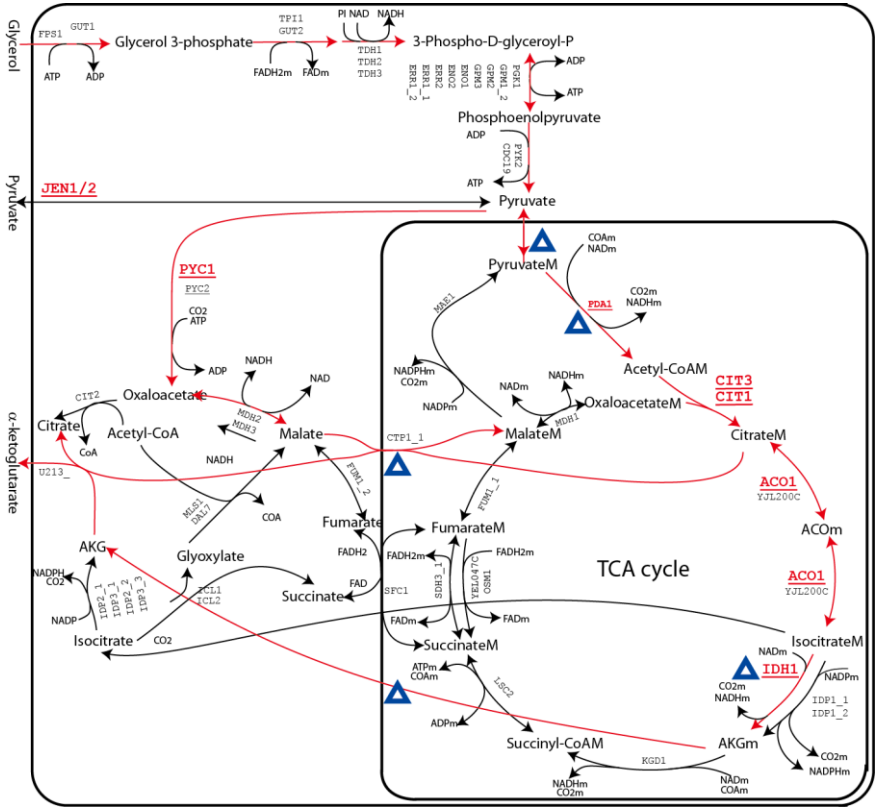


Friedel-Crafts acylation

Production methods-Microbial fermentation

Some keto acids (pyruvic acid, α-ketoglutaric acid) can be produced by fermentation.

- **Advantages: recycle of raw material and low pollution.**
- **Disadvantages: complicated metabolic pathway and low conversion rate.**

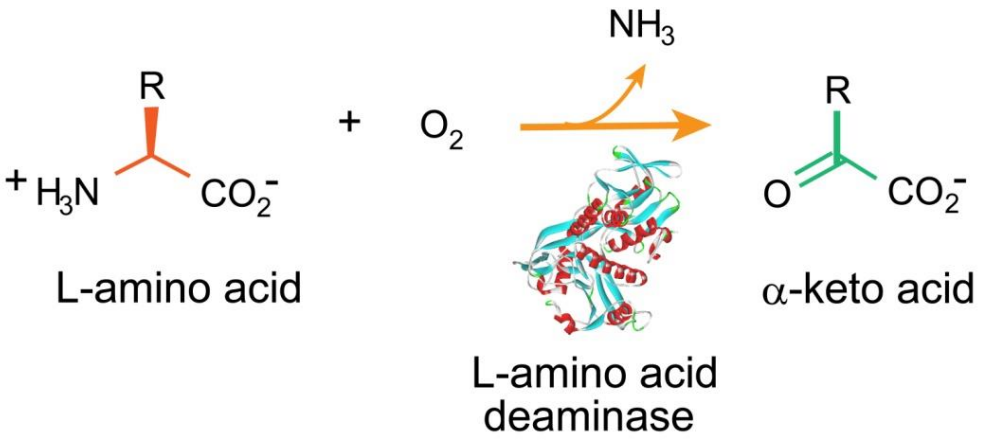


Metabolic pathways of pyruvic acid and α-ketoglutaric acid in *Yarrowia lipolytica*

Production methods-Biotransformation

L-amino acid deaminases (L-AAD) can be used to produce keto acids (α-ketoglutaric acid, α-phenylpyruvic acid) by biotransformation of amino acids.

- **Advantages: high conversion rate (>70%), simple reaction system and low purification costs.**
- **Disadvantages: not very high production because of low substrate tolerance.**



AA	Pm1	LAD [20]	Pma [8]
Ala	9.0	3.5	0.6
Arg	51.2	27.3	28.2
Asn	5.2	43.6	0.0
Asp	2.6	55.4	10.9
Cys	9.0	–	1.9
Gln	5.2	1.1	1.3
Glu	35.8	1.1	0.6
Gly	7.6	–	1.3
His	**100.0**	79.9	0.0
Ilu	6.4	–	2.6
Leu	7.6	**105.0**	41.7
Lys	7.6	3.5	1.9
Met	2.6	**100.0**	16.7
Phe	46.2	37.4	**100.0**
Pro	14.2	0.7	3.2
Ser	3.8	–	1.3
Thr	12.8	1.1	0.0
Trp	10.2	41.6	3.2
Tyr	9.0	92.8	0.6
Val	6.4	–	1.3

For the same amino acid, amino oxidases from different sources have different conversion rates.

3. Biotransformation production of α-keto acids

Example 1: Biotransformation of L-glutamate to α-KG

- α-KG(α-ketoglutaric acid), a rate-determining intermediate in the Krebs cycle, plays crucial roles in cellular energy metabolism, coordinating carbon ,and nitrogen utilization and has a wide range of applications.

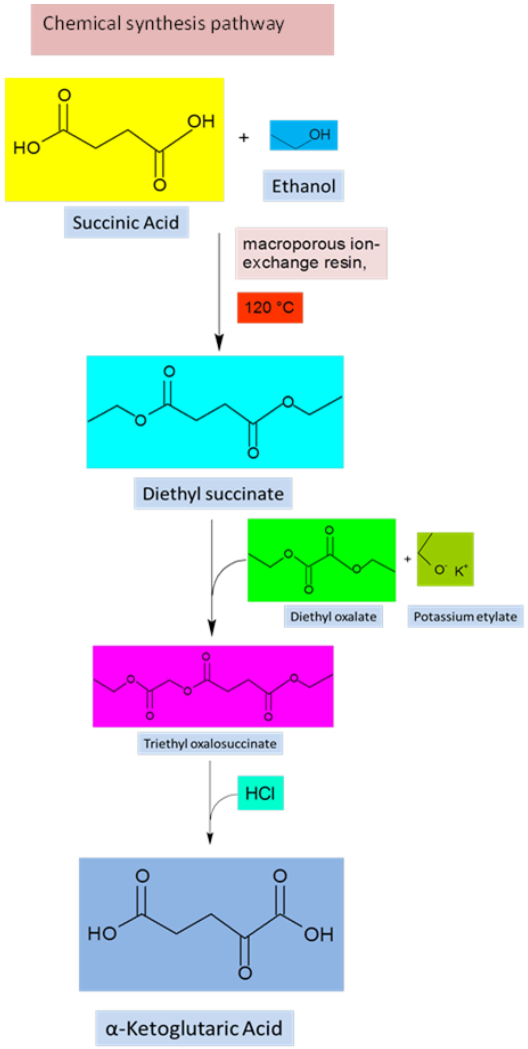
Body reinforcing agent and additives of drink

Free radical scavenging and anti-aging

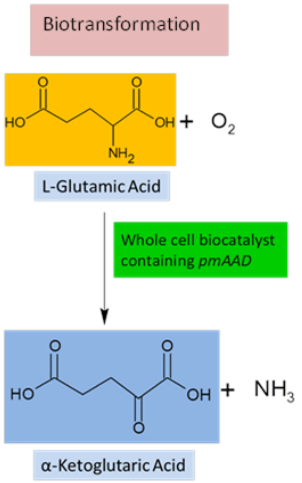
Organic intermediates

Improve reproduction rate and promote bone's growth

Biotransformation of L-glutamate to α-KG



Chemical synthesis



Biotransformation

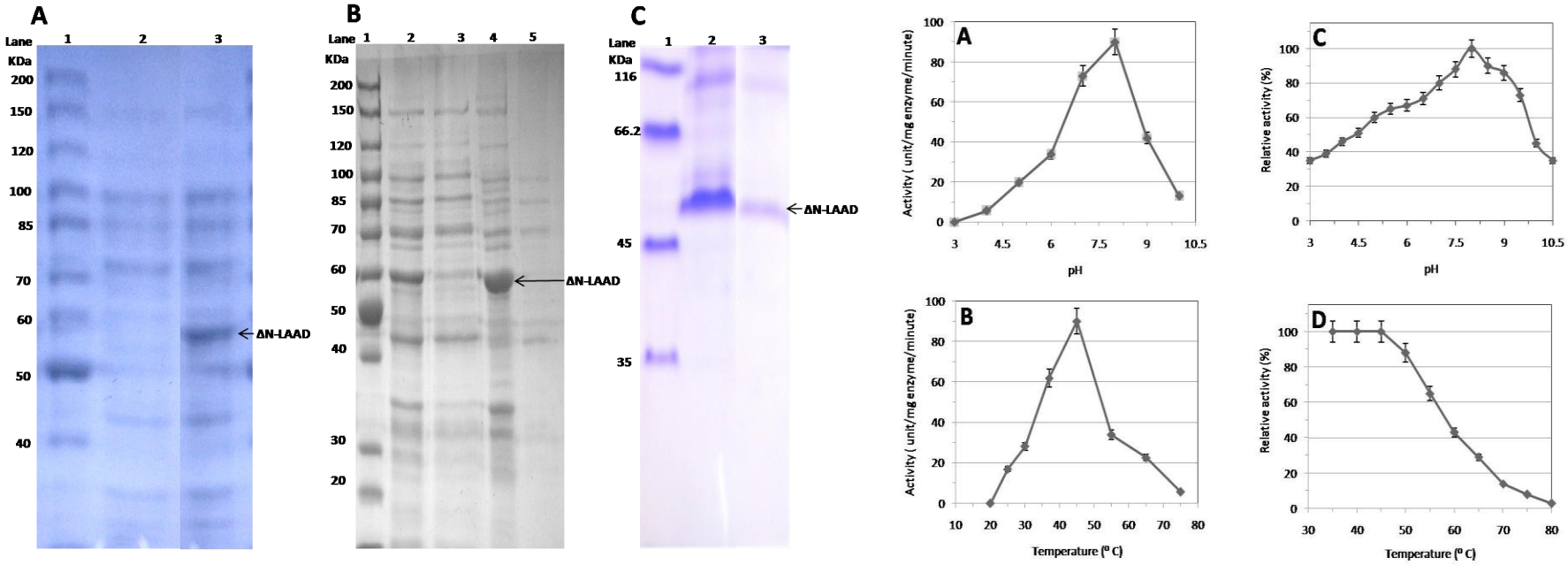
- Currently α-KG is produced mainly by chemical synthesis.

Advantages of biotransformation:

- one-step and high efficiency.
- no toxic catalyst and less pollution.

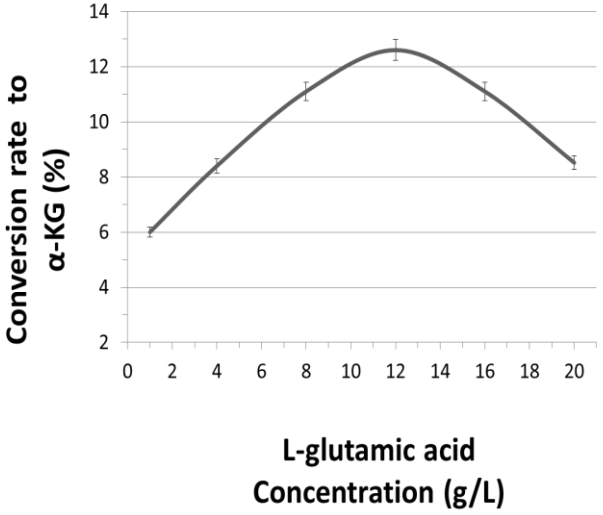
Expression and purification of N-terminus deleted L-AAD

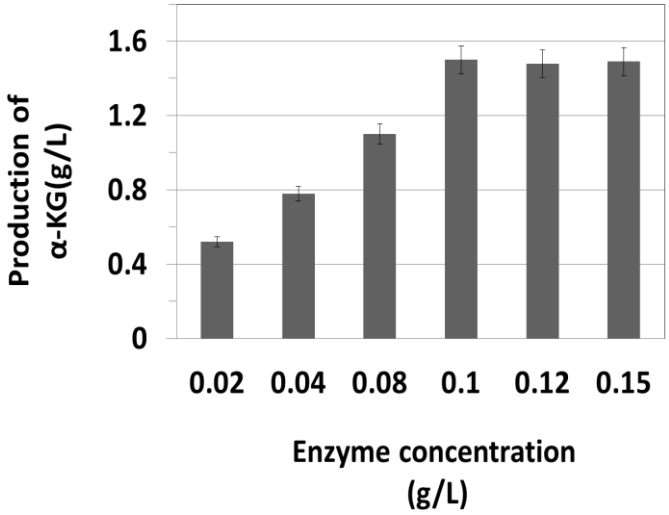
- L-AAD gene was cloned from *Proteus mirabilis*.
- *P. mirabilis* pm1, a transmembrane protein, catalyzes many amino acids to keto acids.
- Compared with amino acid oxidases, L-AAD doesn't need cofactor and no byproduct hydrogen peroxide is produced.

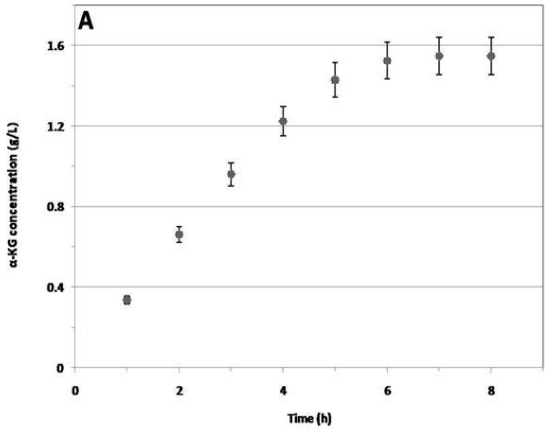


- **To get purified protein, transmembrane regions (21~87 nucleotides) was deleted, resulting in the formation of inclusion bodies.**
- **Refolding of inclusion bodies and the active protein was obtained.**
- **Optimal temperature and pH of the refolded protein: 45°C, pH 8.0.**

Optimization of biotransformation conditions



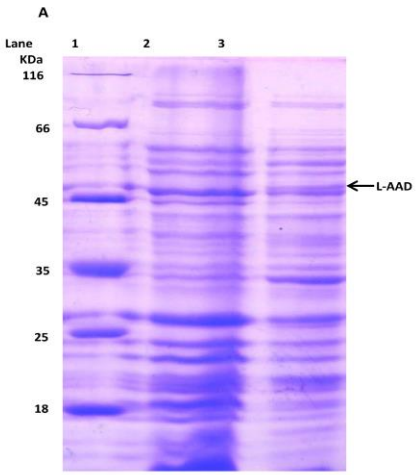


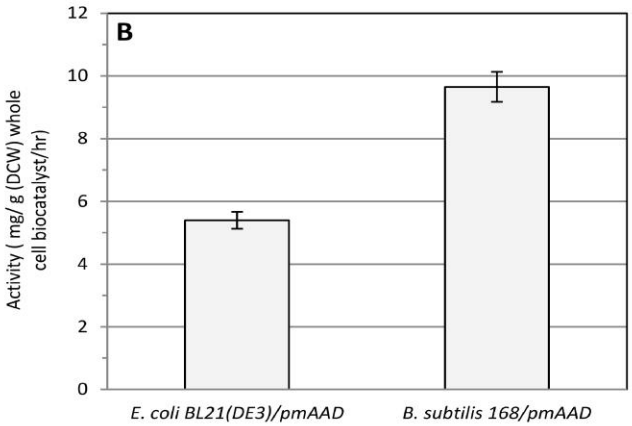


- **Transformation of L-glutamate with the refolding enzymes and the reaction conditions were optimized.**
- **Optimal conditions**: L-glutamate 12g/L, enzyme 0.1 g/L, $MgCl_2$ 5mM, temperature 45 ^oC, and pH 8.0;
- Transformation for **6 h**, the conversion rate is **12.6%** and α-KG production is **1.5 g/L**.

Construction of whole-cell biocatalyst

- **Compared with free enzymes, whole-cell biocatalysts are more convenient to use, less expensive to prepare, and more stable.**
- **Two expression system (*E. coli* and *B. subtilis*) of *P. mirabilis* pm1 was constructed.**
- ***B. subtilis* L-AAD showed higher activity and more suitable for producing α-KG.**

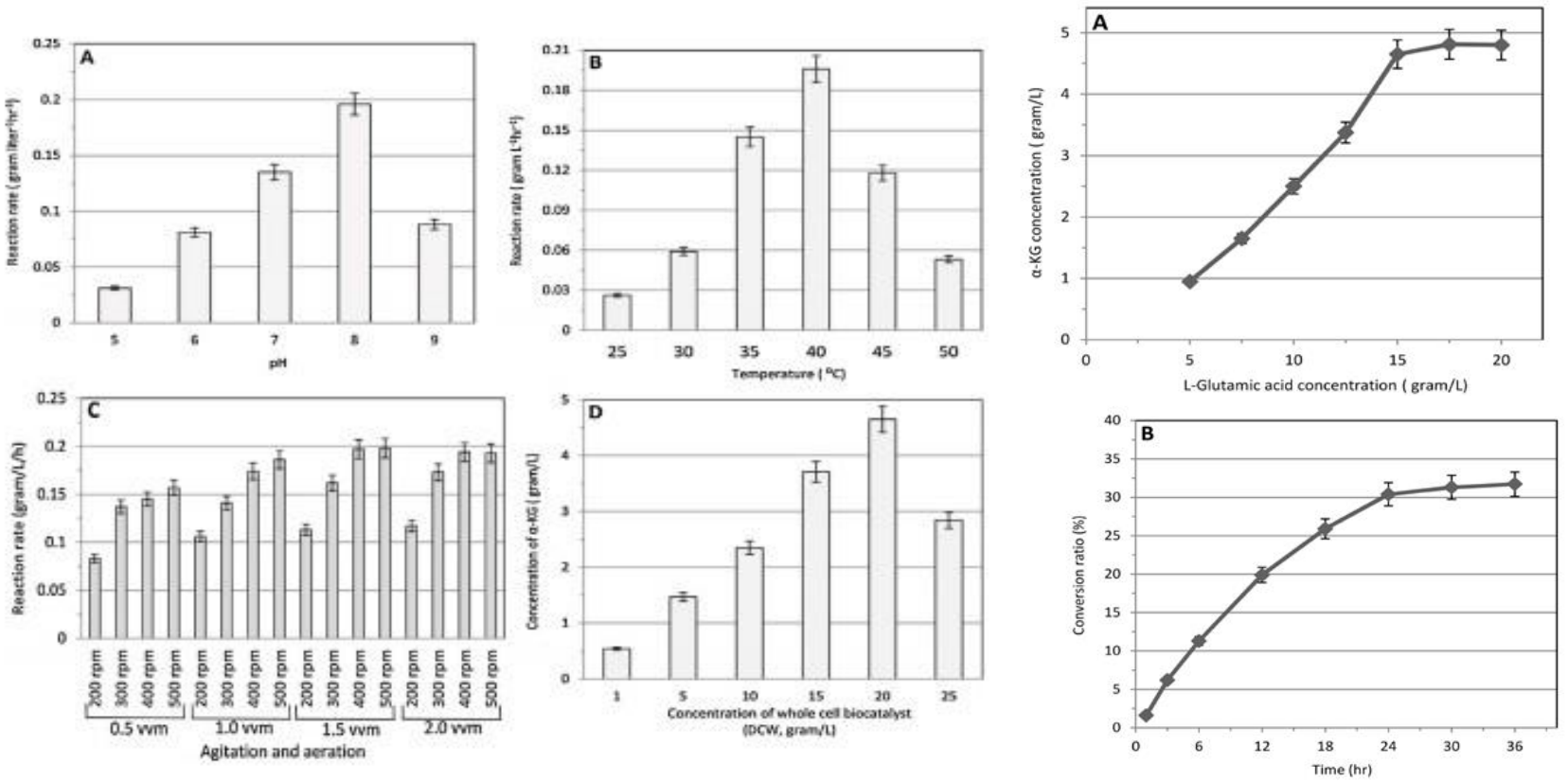




Construct \ Cellular fraction	Amino acid deaminase activity		
	Cultural broth	Cytosol	Membrane
pHT43 (*B. subtilis*168)	ND	3.9±0.08	ND
pHT43-*pmAAD* (*B. subtilis*168)	ND	4.1±0.11	55.3±1.73
pET-20b(+) (*E. coli* BL21)	ND	2.2±0.09	ND
pET-20b(+)-*pmAAD* (*E. coli* BL21)	ND	2.4±0.13	21.7±0.39

Optimization of whole-cell biotransformation of *B. subtilis*

- Optimizing the transformation conditions of *B. subtilis* whole-cell catalyst.
- Optimal conditions: L-glutamate 15g/L, biocatalyst 20 g/L, $MgCl_2$ 5mM, temperature 40 °C, and pH 8.0;
- Biotransformation for **24h**, the conversion rate is **31%** and α-KG titer is **4.7 g/L**.



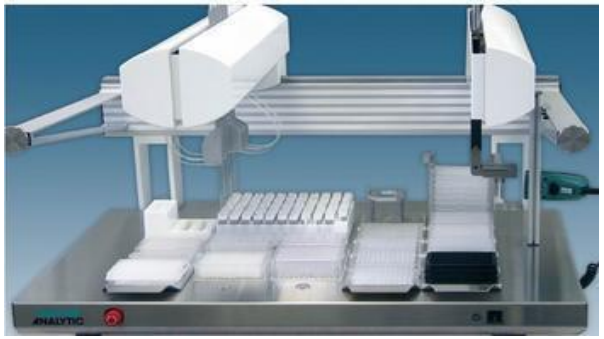
Optimization of transformation conditions

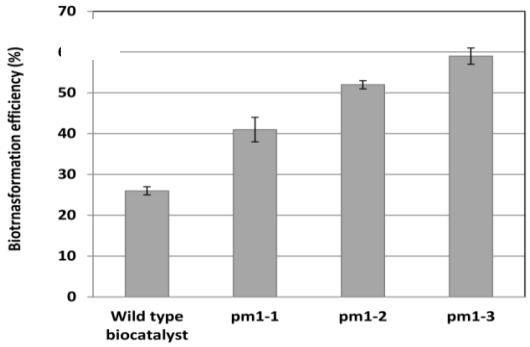
Directed evolution and site-directed mutagenesis of L-AAD

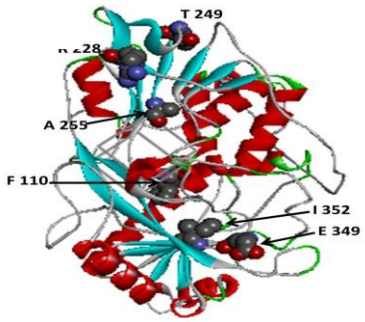
- Three rounds of error prone PCR was performed and the key sites were identified.
- Then site-directed mutation was performed and the optimal mutant F110I/A255T/E31D/R228C L249S/I351T was obtained.

Round of ep-PCR/site-saturated mutant	Mutation presents
First round (pm1-1)	F110I/A255R
Second round (pm1-2)	F110I/A255R**/E31D/R228F**
Third round (pm1-3)	F110I/A255R/E31D/R228F/**T249L/I351T**
Site-saturated-2 (pm1-3-1)	F110I/**R255T**/E31D/R228F/T249L/I351T
Site-saturated-4 (pm1-3-2)	F110I/A255T/E31D/**F228C**/T249L/I351T
Site-saturated-5 (pm1-3-3)	**F110I/A255T/E31D/R228C/L249S/I351T**

- F110I/A255T/E31D/R228C/L249S/I351T exhibited 57.2% of conversion rate and 8.6 g/L of α-KG production.





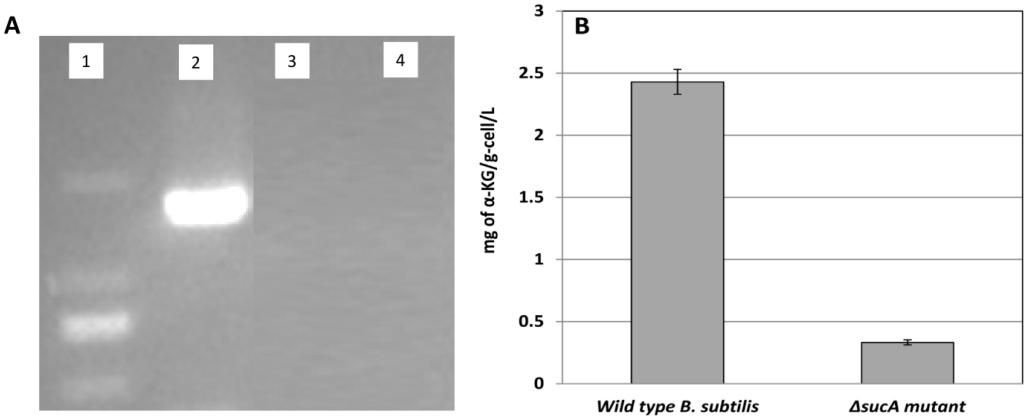


Directed evolution, site-directed mutagenesis, and modeling of L-AAD based on high throughput screening

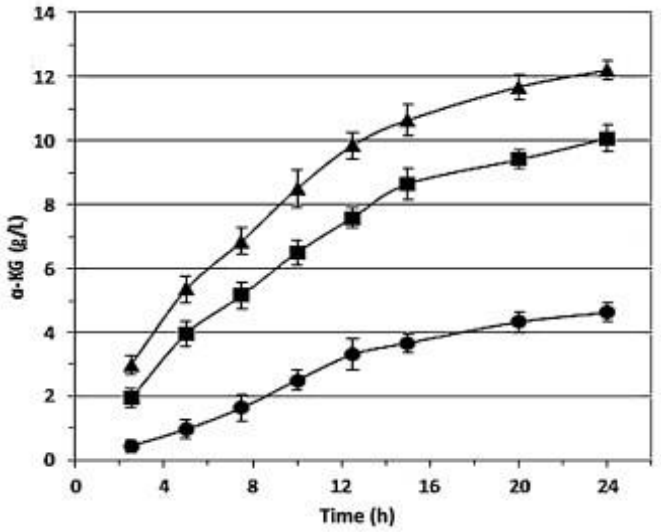
Deletion of α-KG dehydrogenase to reduce its degradation

- α-KG dehydrogenase gene (*SucA*) was knocked out, the conversion rate and production of α-KG were improved to 85.3% and 12.2 g/L, respectively.

Strains/Mutants	Km (mM)	Vmax (min^{-1}uM)	K_{cat} (min^{-1})	K_{cat}/K_m ($uM^{-1}min^{-1}$)
Wild type	49.21+0.05	22.82±0.08	0.812	60.61
pm1-1	41.42+0.04	32.48±0.08	0.859	48.21
pm1-2	38.91+0.03	36.45±0.09	0.83	46.88
pm1-3	34.12+0.01	40.76±0.04	0.839	40.66



Electrophoresis map for deletion of *SucA* and the uptake of α-KG by mutant strain

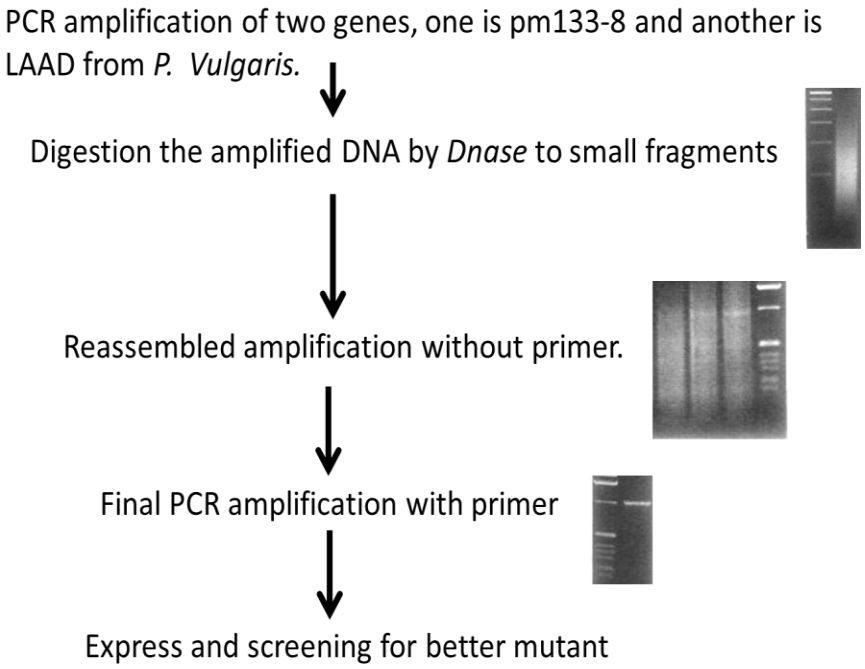


Time profile for the biotransforamtion of L-glutamic acid to α-KG by wild-type, engineered and mutant whole cell biocatalyst

Gene shuffling and error-prone PCR of L-AAD were used to improve the biotransformation of glutamate to α-KG

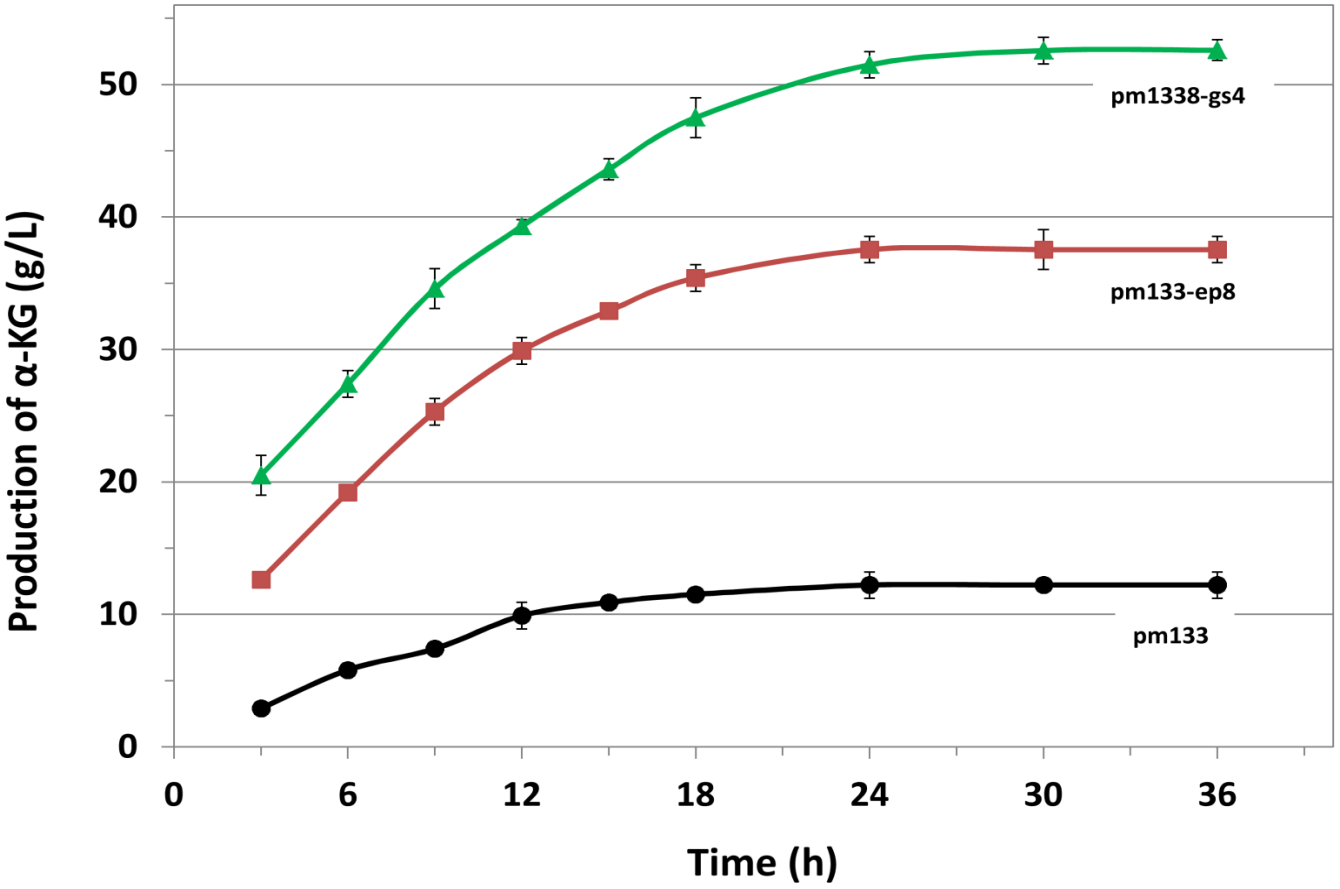
Mutation occurred in the L-AAD after eight round of ep-PCR and three round of gene shuffling experiments	G259W/D362N/N150K/ Q278L/ G437V / G193A / P320S / P246A / D374V / D340E / V271I / V445A / A295H / P415F / E383H / D147A / I317F / G291R /S408G/E366K/N418/V269I/E400K/P275N/V258I/L378T/L267M/ E389Q/A285G/A286V/ R251Q

Strategy for the evolution of L-amino acid deaminase (pm133-8) by gene shuffling with LAAD from *P. vulgaris*:



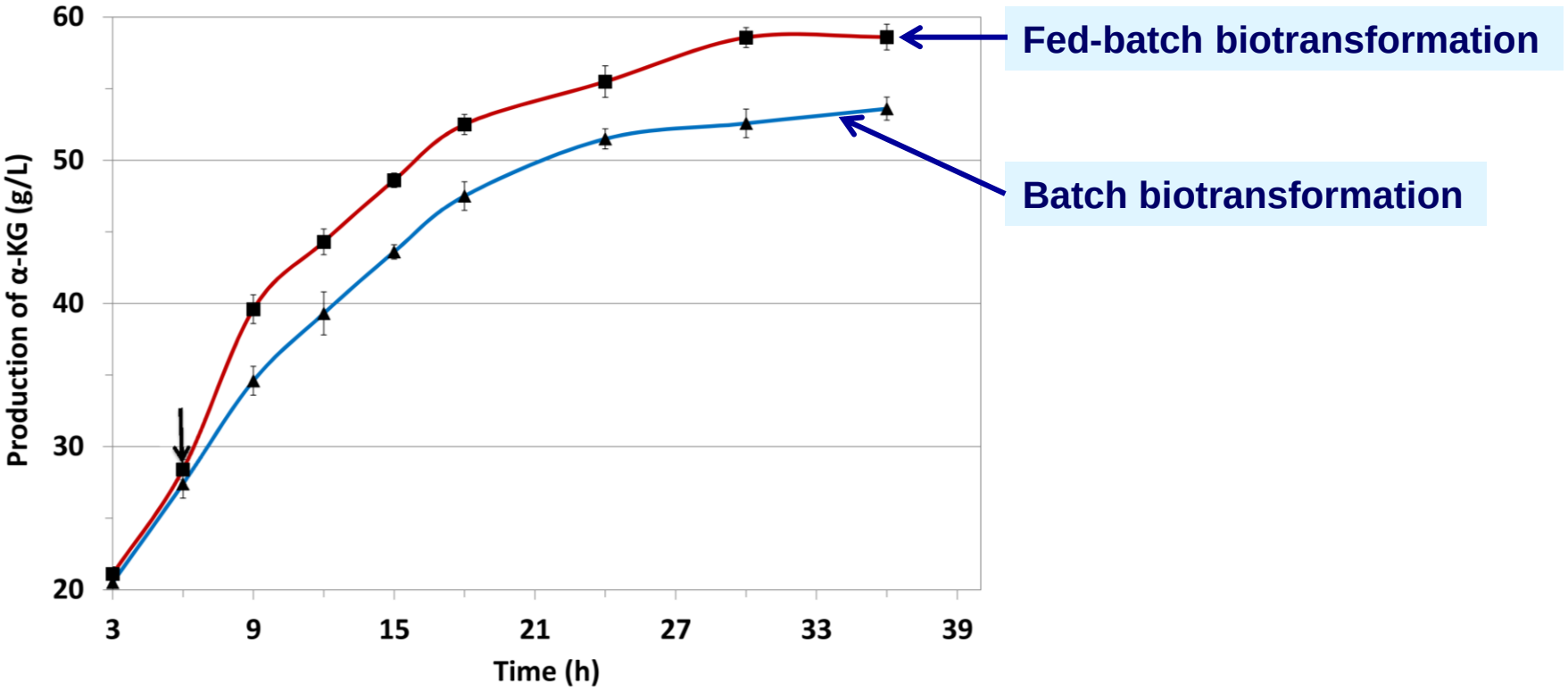
Mutants	Vmax (μM min^{-1})	Km (mM)	Vmax/Km (min^{-1})
Pm133	56.7 ± 1.11	23.58 ± 0.97	0.0024
Pm133-ep1	79.2 ± 0.92	21.32 ± 1.78	0.0037
Pm133-ep2	92.6 ± 1.34	18.11 ± 0.69	0.0051
Pm133-ep3	105.3 ± 1.65	16.72 ± 0.82	0.0063
Pm133-ep4	142.7 ± 1.52	13.26± 0.46	0.0107
Pm133-ep5	168.8 ± 1.09	11.69 ± 0.23	0.0144
Pm133-ep6	159.1 ± 1.31	10.53 ± 0.32	0.0151
Pm133-ep7	155.9 ± 1.11	10.17 ± 0.13	0.0153
Pm133-ep8	167.2 ± 0.81	8.83 ± 0.17	0.0189
Pm1338-gs1	184.6 ± 0.43	8.12 ± 0.09	0.0226
Pm133-gs2	207.1 ± 0.57	7.54 ± 0.12	0.0274
Pm133-gs3	223.8 ± 0.32	6.91 ± 0.23	0.0322

Biotransformation of glutamate to α-KG by mutant L-AAD containing biocatalysts in the flask



● By gene shuffling and error-prone PCR of L-AAD, α-KG production was improved to 52.7 g/L.

Biotransformation of glutamate to α-KG by mutant L-AAD containing biocatalysts in the 3L fermenter



- The conversion rate was about 67.7% and α-KG production was improved to 58.6 g/L in the fed-batch system.

Example 2: Biotransformation of L-phenylalanine to PPA

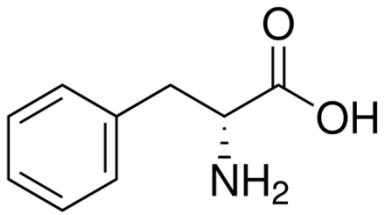
- Phenylpyruvic acid (PPA) is widely used in the pharmaceutical, food, and chemical industries.

Pharmaceutical intermediates

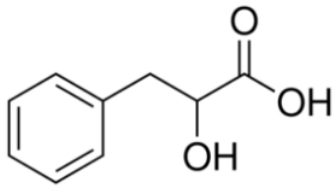
Food

Fine chemistry

Animal feeding



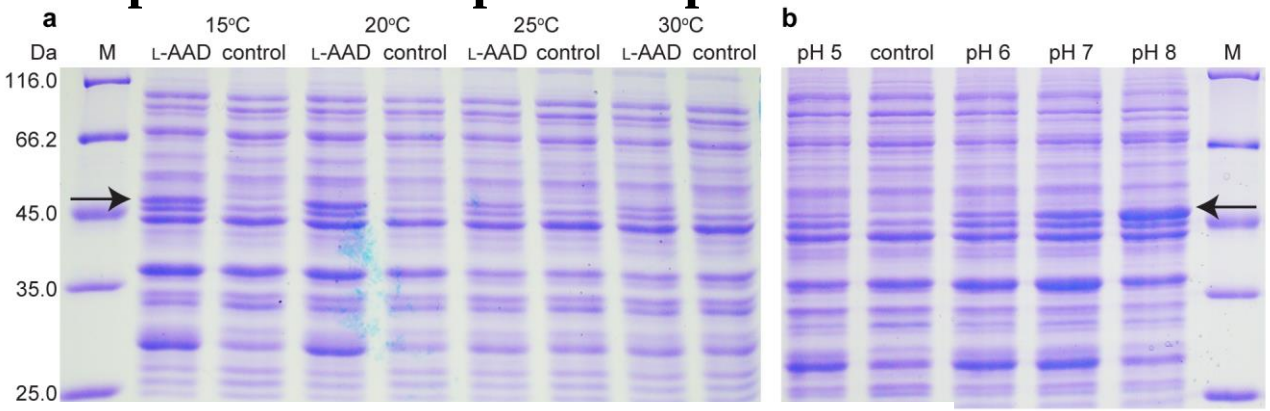
D-phenylalanine

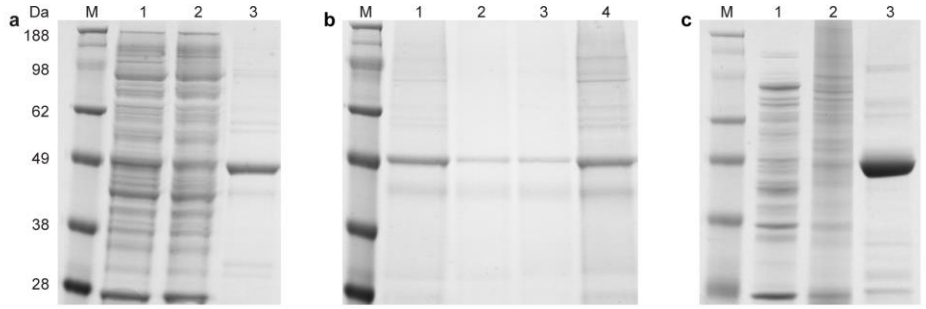


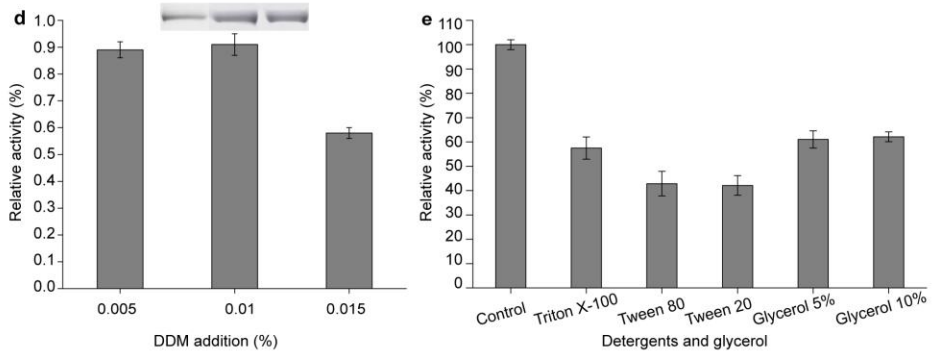
Phenyllactic acid

Expression and purification of the L-AAD

- **L-AAD gene from *P. mirabilis* were expressed and induction conditions and purification steps were optimized.**



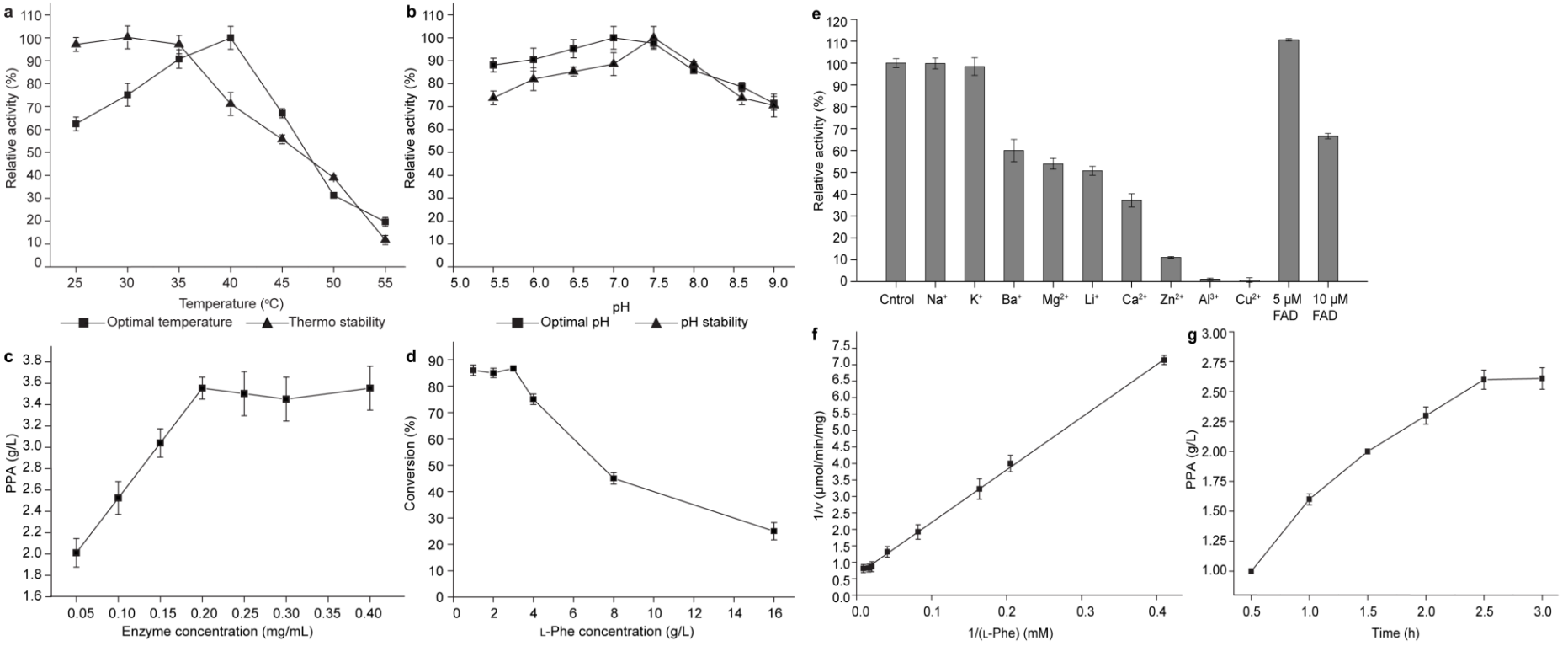




- The optimal induction conditions: pH 8, 0.04 mM IPTG, OD_{600} 0.6, and induction at 20 °C for 12 h.
- The enzyme was purified **52-fold**, with an overall yield of **13%**, corresponding to a specific activity of 0.94 µmol PPA min/mg protein

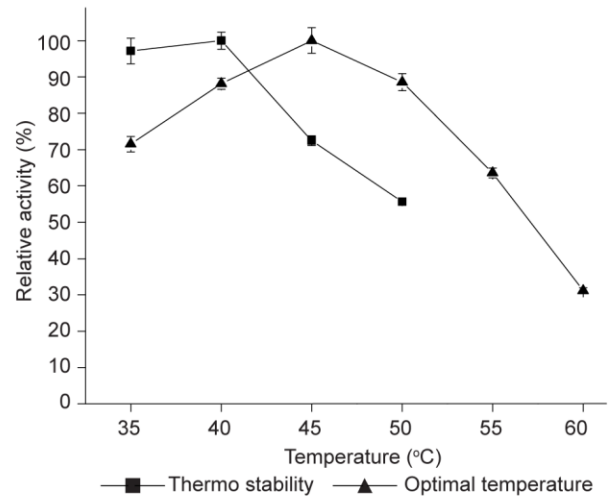
Optimization of enzymatic biotransformation

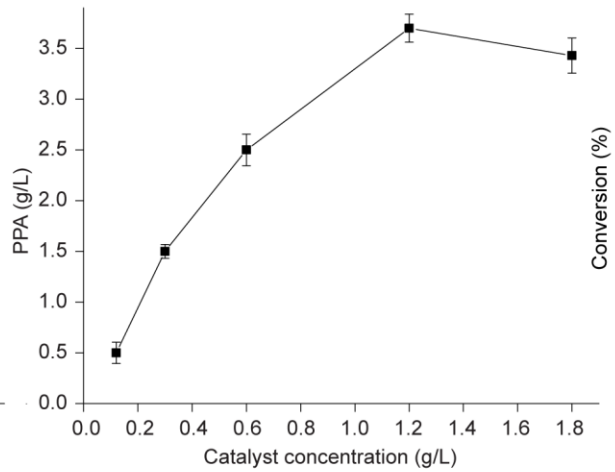
- **Characterization of L-AAD and enzymatic transformation**

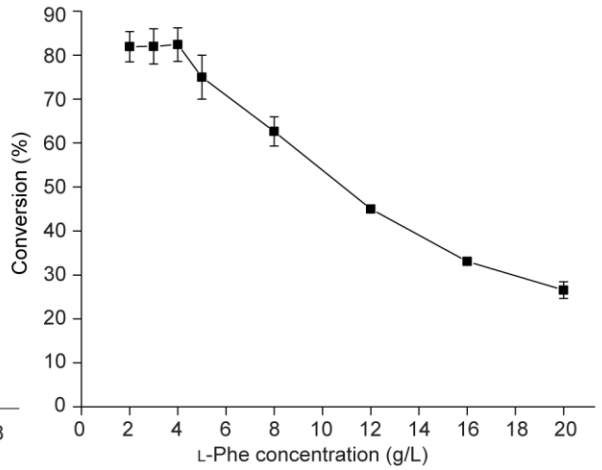


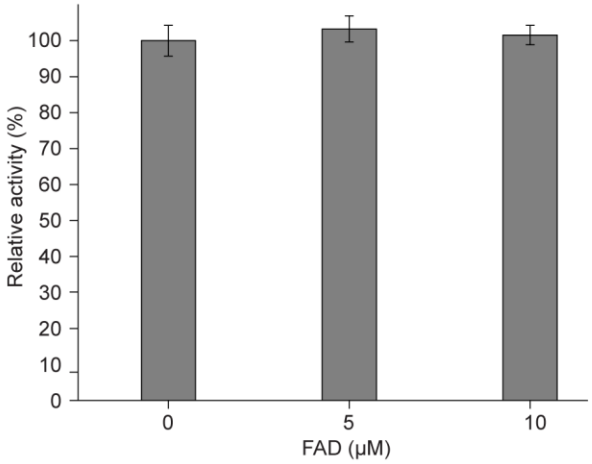
● The maximal conversion rate and PPA titer reached **86.7%** and **2.6 g/L** at **2.5 h**: 0.2 g/L L-AAD, 3 g/L of L-phenylalanine, 5 mM FAD, 35 °C and pH 7.4.

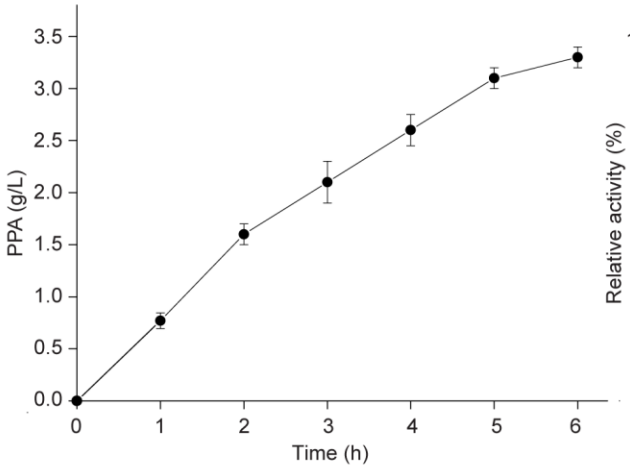
Whole-cell transformation system

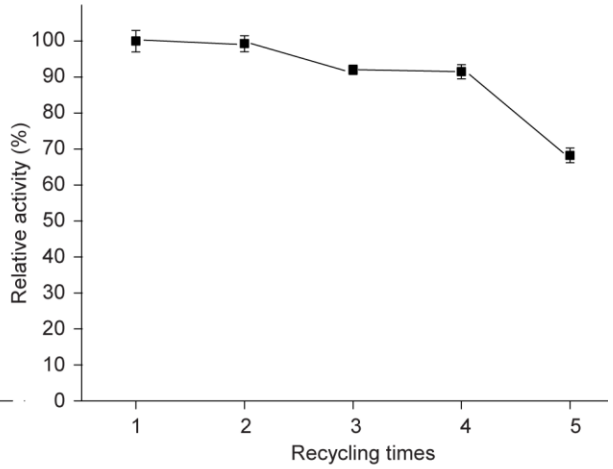








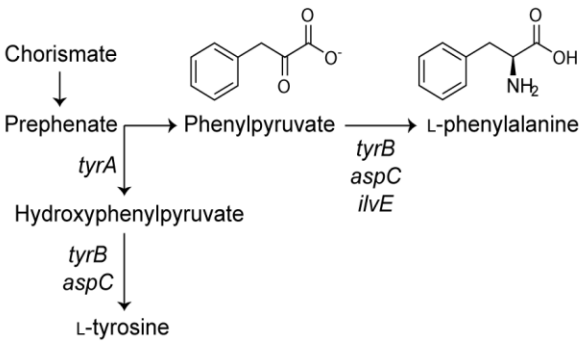


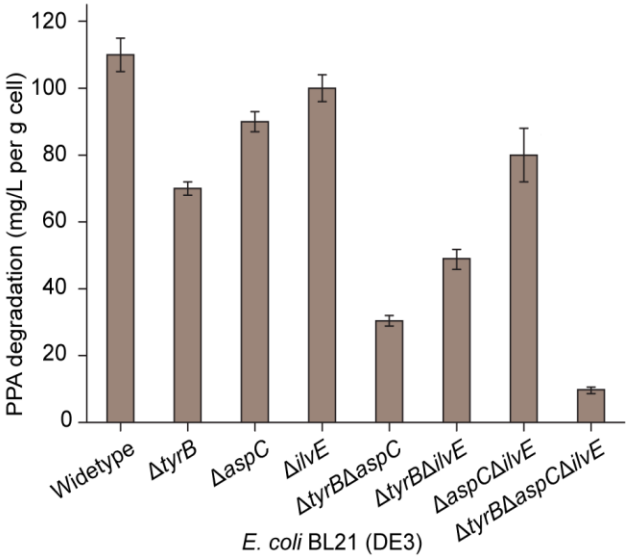


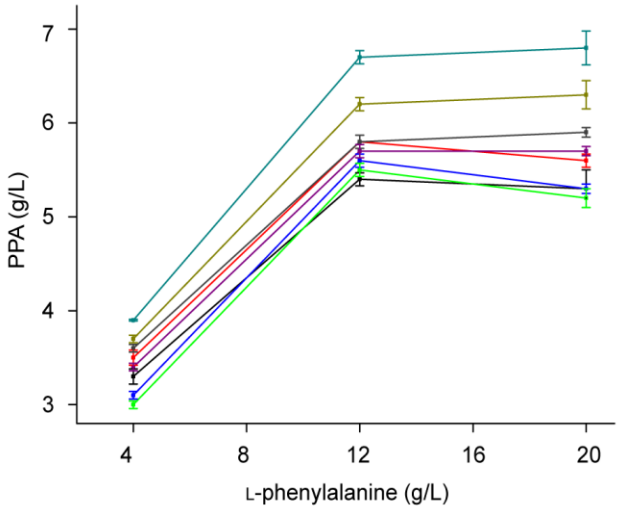
●The maximal conversion rate and PPA titer reached **82.5%** and **3.3 g/L** at **6 h**: 1.2 g/L of biocatalyst, 4 g/L of L-phenylalanine, 40 °C and pH 7.4.

Metabolic engineering of to delete PPA degradation pathway in *E. coli*

- Three aminotransferases participate in the degradation of PPA
- Single-, double-, and triple-deletion mutants were constructed in *E. coli* BL21 (DE3) to determine the amount of PPA degradation



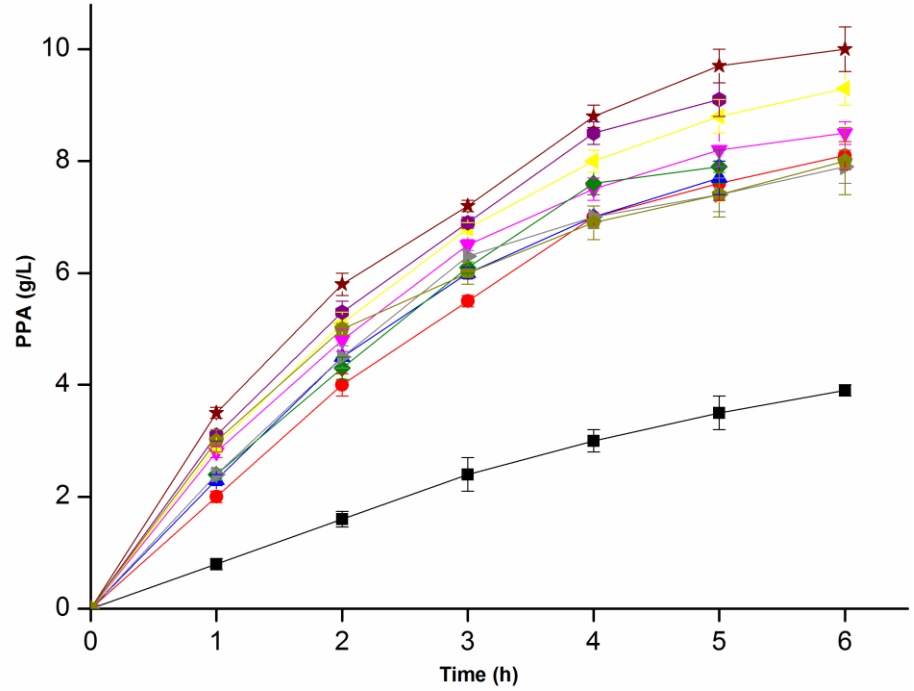




●For the triple-deletion mutant *E. coli* BL21 (DE3) (Δ*tyrB*Δ*aspC*Δ*ilvE*), and the PPA titer was improved to 3.9 g/L.

Directed evolution and site-directed mutation of L-AAD

- Two rounds of error prone PCR was performed and the key sites were identified.
- Then site-directed mutation was performed and the mutant D165K/F263M/L336M was obtained.

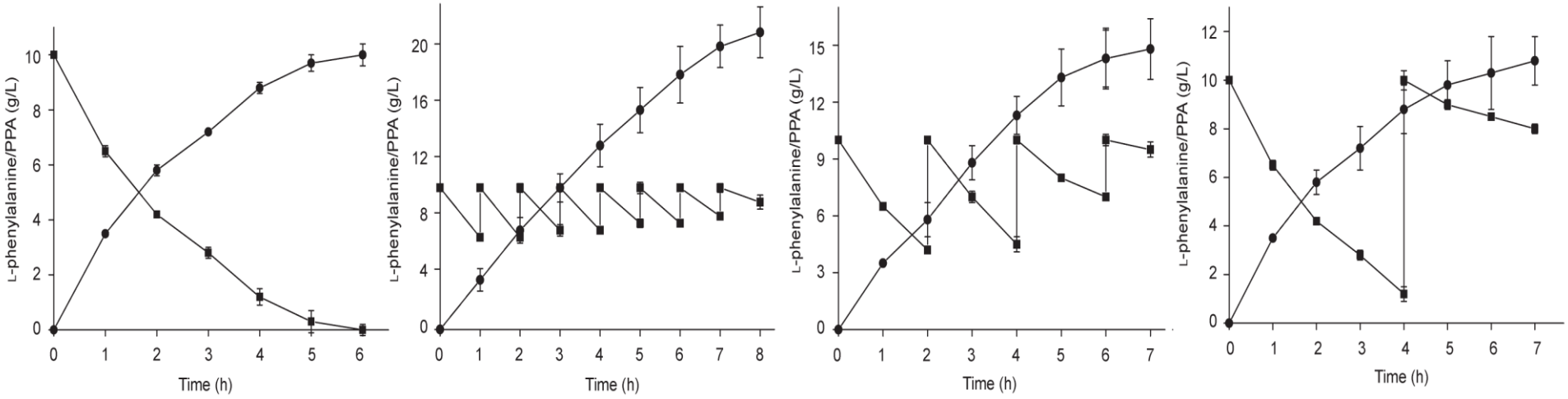


Enzymes	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1}M^{-1}$)
Wild type	26.2±0.1	1.40	53
D165G/S179L/F263V/L336V	24.1±0.4	1.82	76
D165K	24.8±0.8	1.67	67
L336M	24.5±0.9	1.69	69
F263M	23.8±0.7	1.87	79
D165K/F263M	22.6±0.4	2.16	96
D165K/L336M	24.3±0.9	1.72	71
F263M/L336M	22.4±0.4	2.21	99
D165K/F263M/L336M	22.0±0.9	2.25	102

- The triple mutant D165K/F263M/L336M produced the highest PPA titer of 10.0 g/L with a conversion ratio of 100%.
- Kinetics analysis showed that the triple mutant had a higher substrate-binding affinity and catalytic efficiency than that of wild type.

Fed-batch biotransformation in flask

- Maintain the L-phenylalanine concentration below 10 g/L.
- Beginning with 10 g/L of L-phenylalanine, a specific amount of L-phenylalanine was added and the feeding interval was optimized.

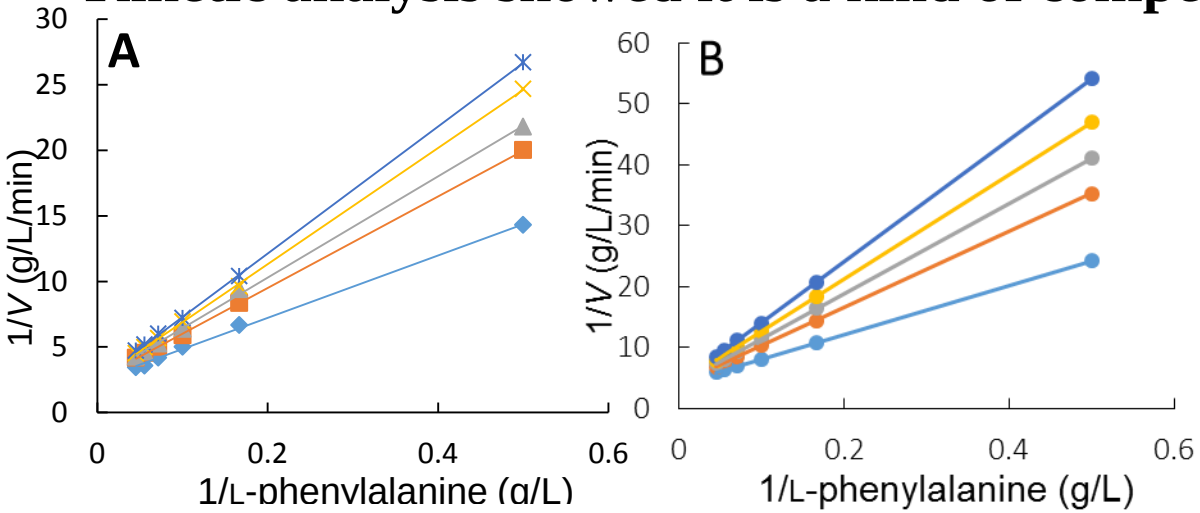


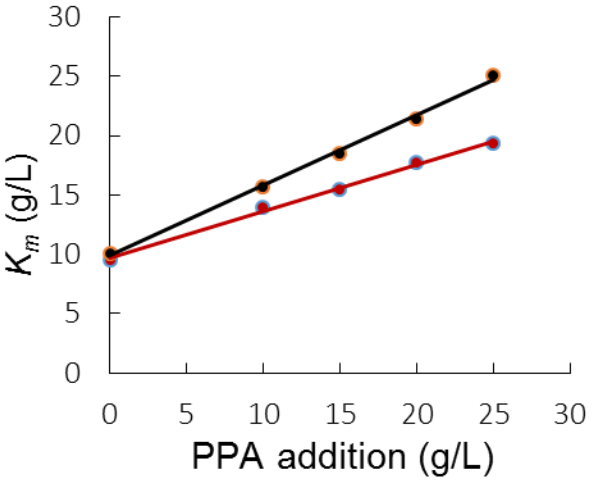
●By feeding the substrate every hour, the maximal PPA production was 21 g/L within 8 h with the total L-phenylalanine at 31 g/L.

Effect of PPA addition on initial rate

To determine of product inhibition constant, different concentration of PPA was added to the biotransformation system.

Kinetic analysis showed it is a kind of competitive inhibition.





0 g/L PPA　10 g/L PPA　15 g/L PPA
20 g/L PPA　25 g/L PPA

A, the engineered recombinant *E. coli*.
B, the wild type recombinant *E. coli*.

Effect of PPA addition on initial rate

— the engineered recombinant *E. coli*.
— the wild type recombinant *E. coli*.

- **Competitive inhibition**

$$\frac{d[P]}{dt} \propto \frac{[S]}{K_m\left(1+\frac{[P]}{K_{PI}}\right)+[S]}$$

Development of model based on initial rate studies

General rate equation for PPA production

$$\frac{\mathrm{d}[P]}{\mathrm{d}t}\Big|_i = V_{\mathrm{P}}\left[\frac{[S_i]}{K_{\mathrm{m}}\left(1+\frac{[P_i]}{K_{\mathrm{PI}}}\right)+[S_i]\left(1+\frac{[S_i]}{K_{\mathrm{SI}}}\right)}\right][Ei]$$

Consumption of L-phenylalanine

$$\frac{\mathrm{d}[S]}{\mathrm{d}t}\Big|_i = -\frac{\mathrm{d}[P]}{\mathrm{d}t}\Big|_i$$

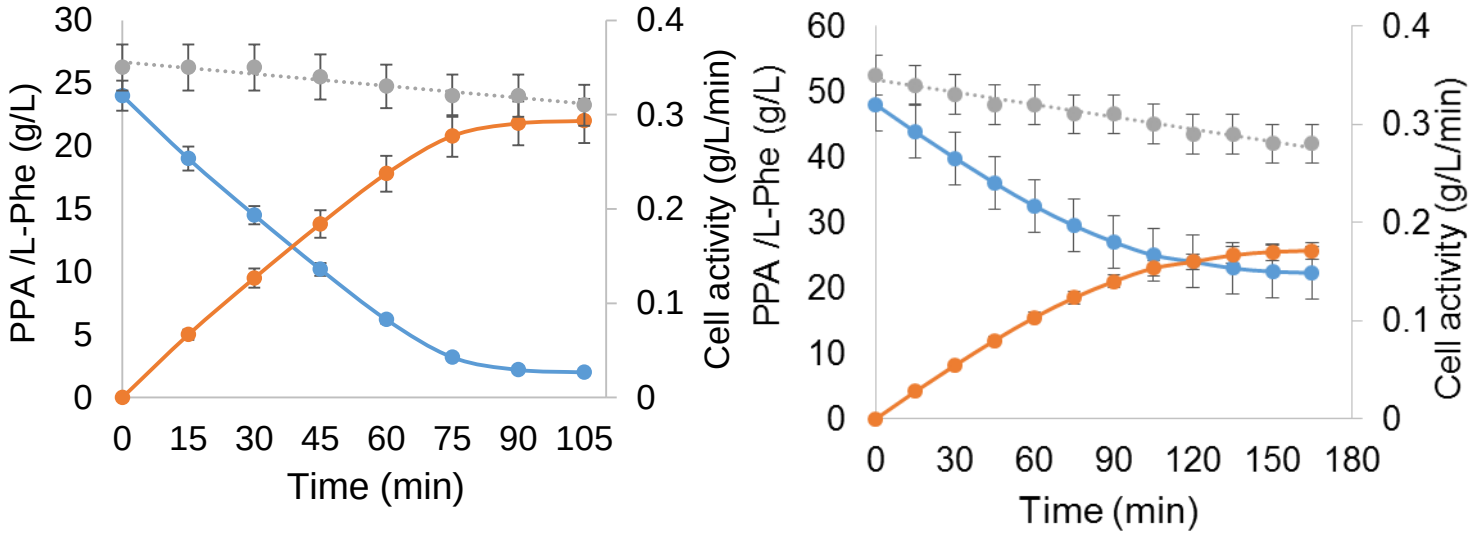
Consumption of the biocatalyst

$$\frac{\mathrm{d}[E]}{\mathrm{d}t}\Big|_i = -k_{\mathrm{d}[E_i]}$$

k_{d}-deactivation constant

Experimental determination of rate constants

Batch biotransformation kinetics and model fitting for determination of rate constants

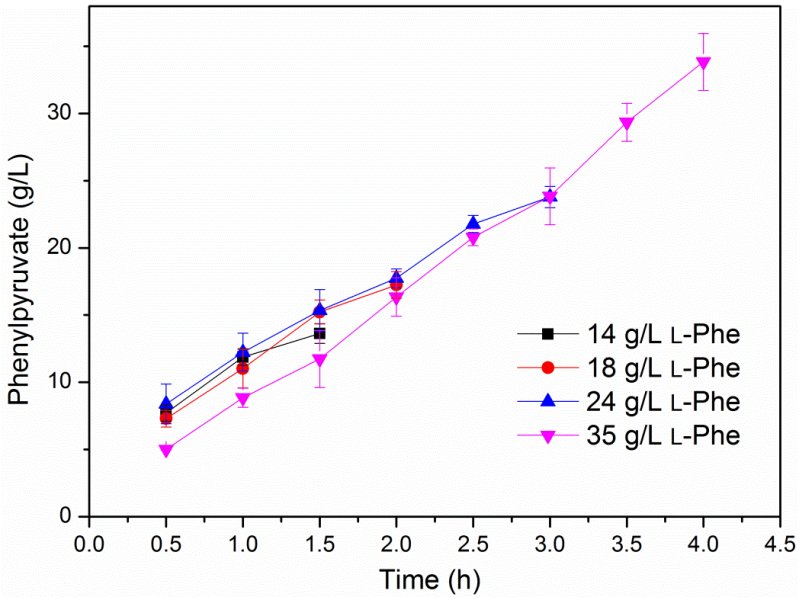


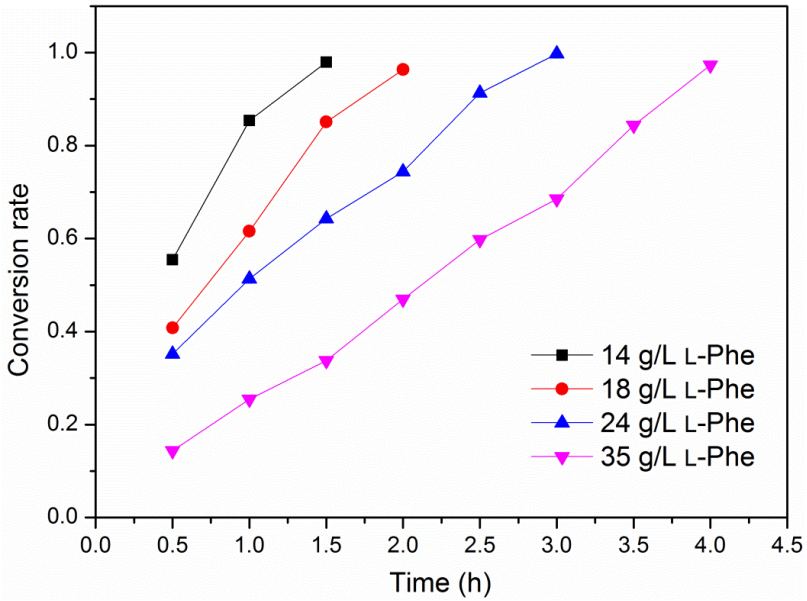
Constants	V_P (g/L/min)	K_{SI} (g/L)	K_{PI} (g/L)	k_d (g/L/min)
Wild type strain	0.21	46.44	16.74	1.26×10^{-3}
Engineered strain	0.35	70.57	24.58	1.47×10^{-3}

- **The substrate and product inhibition of the engineered *E. coli* were 65.8% and 68.1% that of the wild type strain.**

Biotransformation in 3-L fermentor

Whole-cell biotransformation in 3-L fermentor with different concentrations of L-Phe





- **The conversion rate reached 100% within 4 h and no substrate inhibition was observed within 35 g/L of L-Phe.**
- **Improved dissolved oxygen speeds up the oxidative reaction and increases the productivity.**

Example 3: Biotransformation of leucine to α-ketoisocaproate

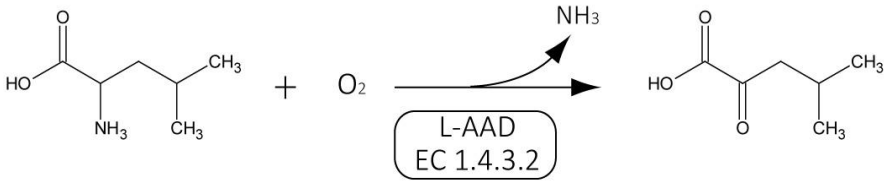
- α-ketoisocaproate (KIC) is widely used in the pharmaceutical, health product and feed.

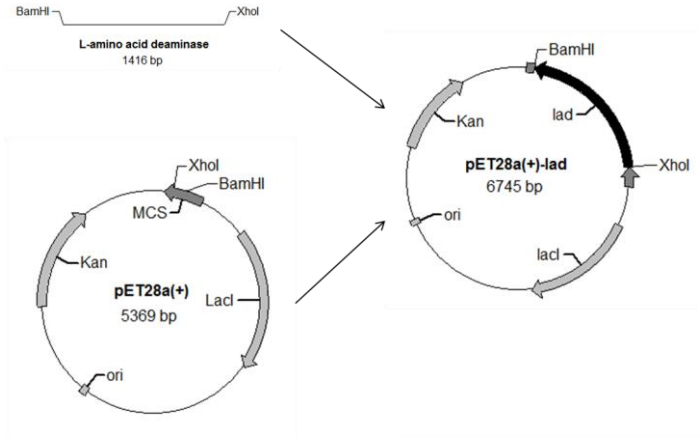
- **KIC could serve as an integral part of therapy for chronic kidney disease to provide daily requirement of L-leucine.**

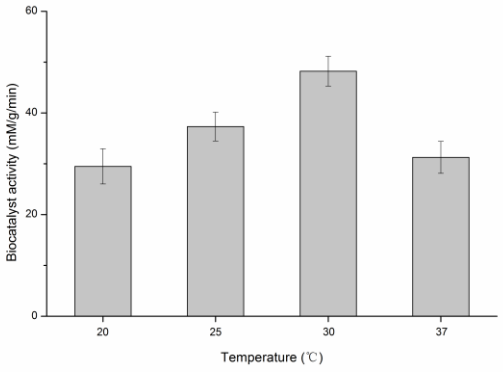
- **KIC could used as the the supplement for the weigh-control or in the physical training program. It has the capacity stimulate protein synthesis, and promote insulin secretion.**

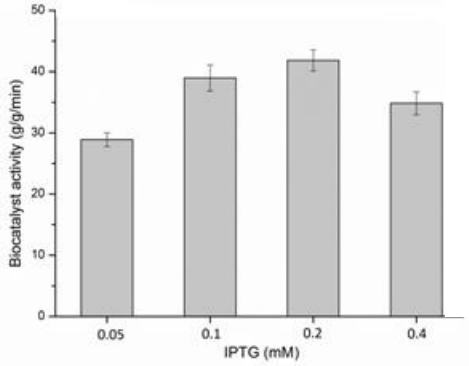
- **In feed, KIC can promote the milk production and composition in cows, goats, and chickens.**

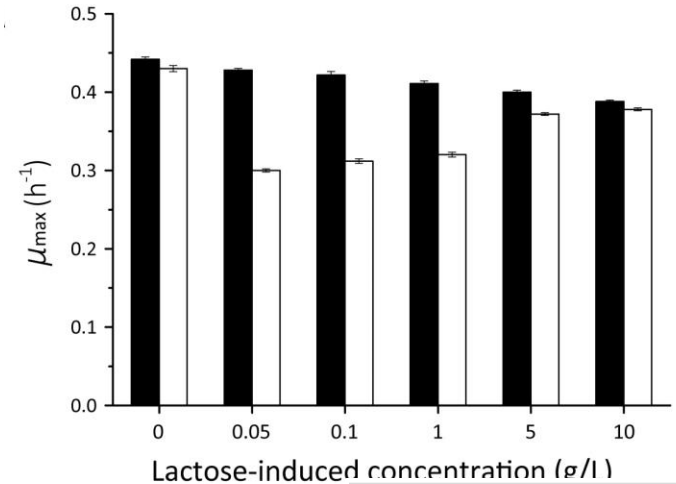
α-ketoisocaproate production by transformation of leucine

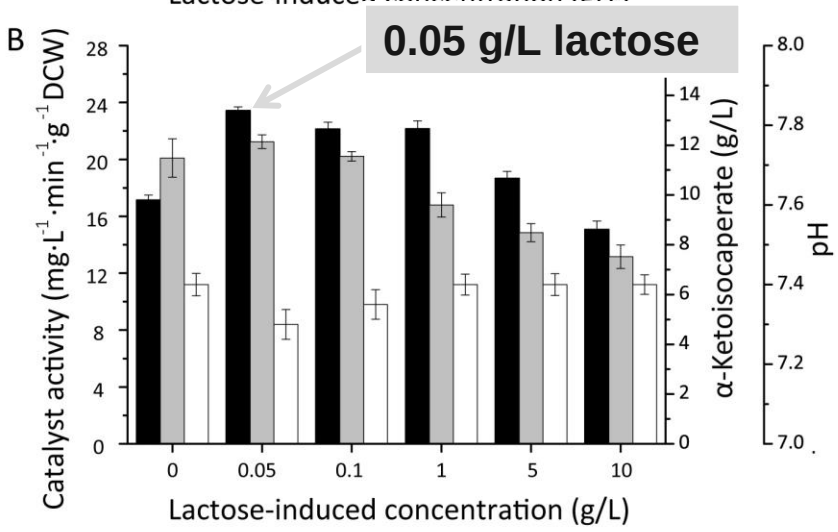






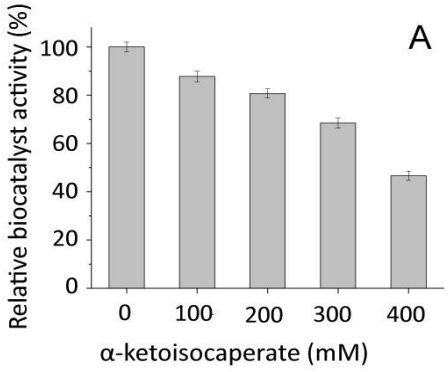


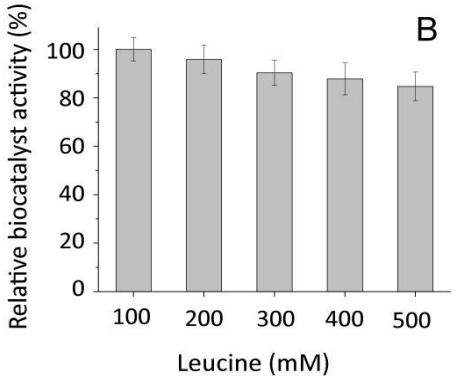


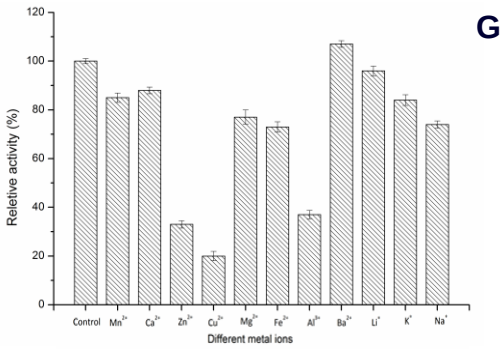


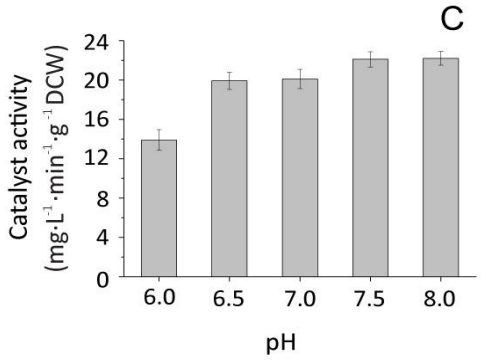
Expression of L-AAD from *P. vulgaris* in *E. coli*

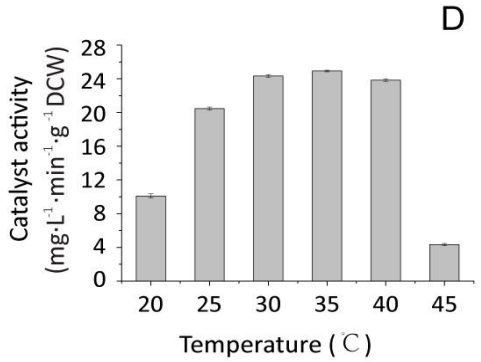
Optimization of KIC production by the whole-cell biocatalyst



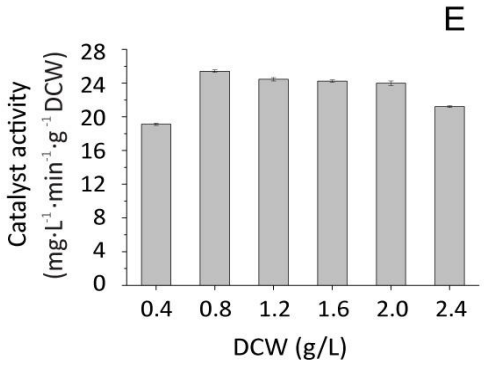


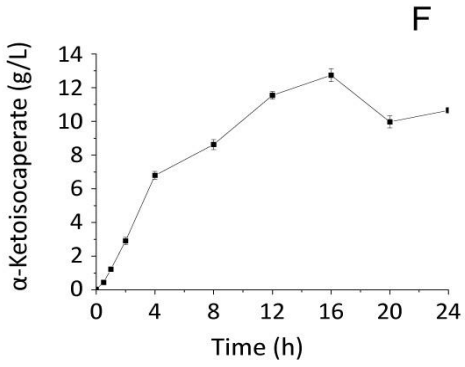






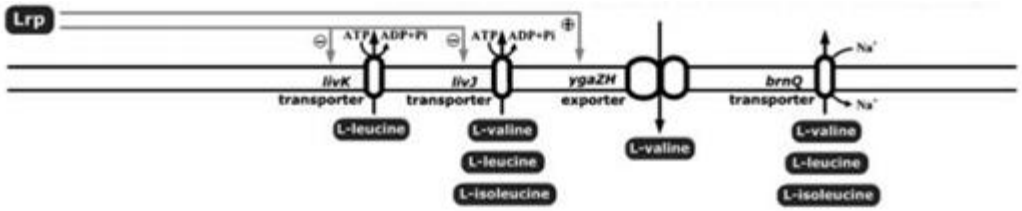
$CaCO_3$ (g/L)	pH	DCW (g/L)	KIC (g/L)
10	6.94	0.44	10.47
15	6.43	0.52	11.92
20	6.39	0.58	**12.69**
25	6.77	0.55	10.60
30	6.83	0.44	9.48
35	7.22	0.33	8.52





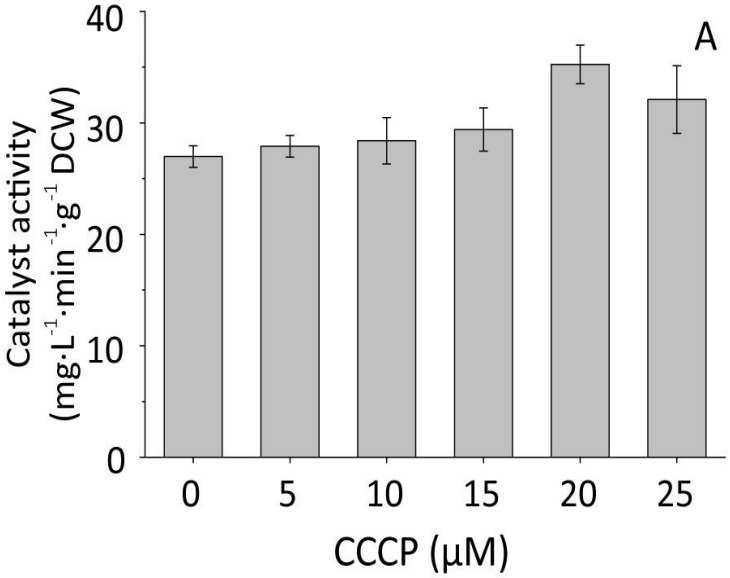
- **The optimal conditions were 0.8 g/L cells, 20 g/L $CaCO_3$ at 35℃ for 16 h.**
- **The conversion rate was 97.8% with 13.1 g/L leucine.**

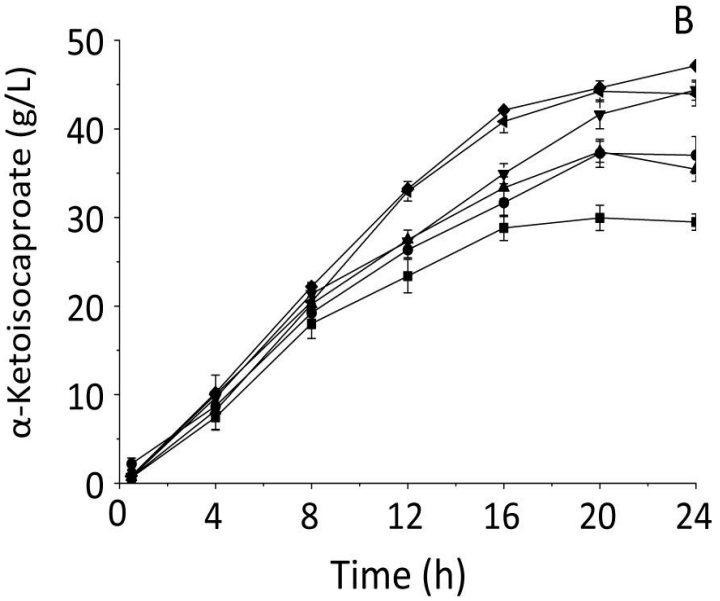
Effect of L-leucine transporters on whole-cell biocatalyst activity



- Carbonyl cyanide-3-chlorophenylhydrazone (CCCP) was used to uncouple the agents that catalyze electrogenic proton movement, in order to reduce ATP generation and inhibit livK, livJ, and BrnQ transport efficiency.

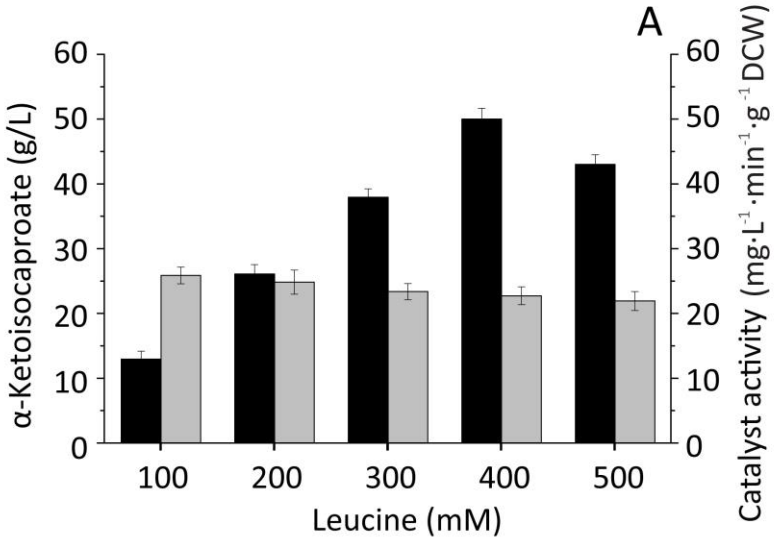
- CCCP increased the biocatalyst activity at concentrations below 20 mM, and the highest KIC production reached 47.1 g/L with 400 mM leucine.



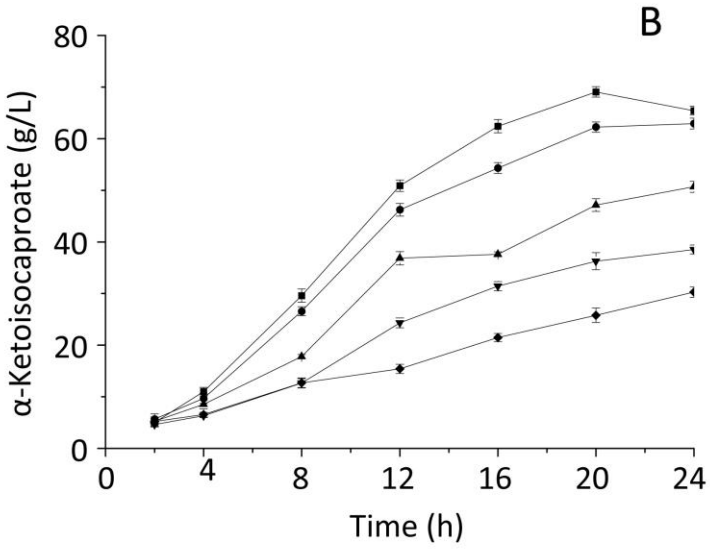


Effect of different L-leucine supply strategies on KIC production

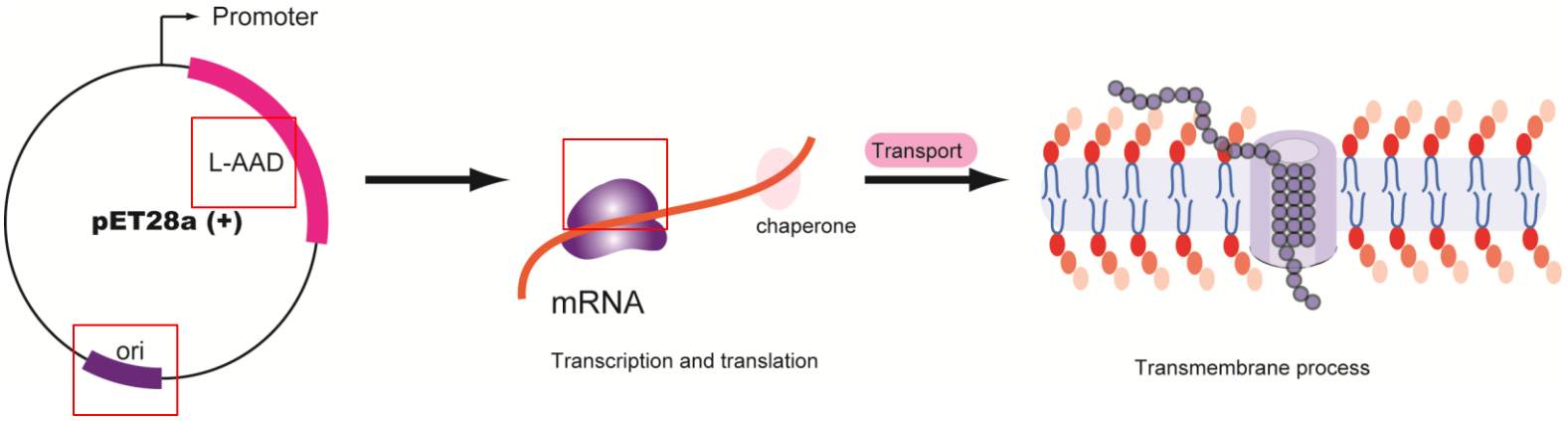
- Batch and interval leucine feeding on KIC production were studied in flask.



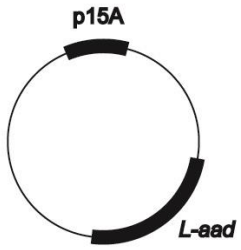
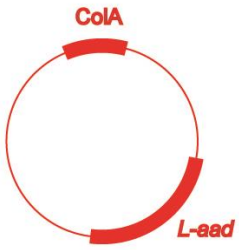
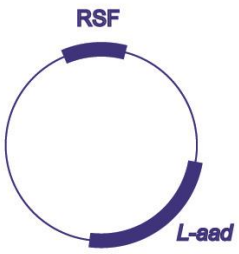
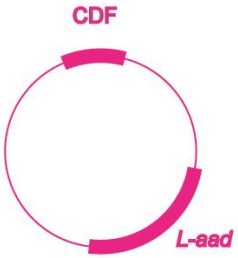
- In batch biotransformation KIC production reached 50.0 g/L with a leucine conversion rate of 96.1% (Fig. A).
- By the feeding of leucine at 2-h intervals (from 0 to 22 h), the KIC titer reached 69.1 g/L when while the leucine bioconversion rate decreased to 50.3% (Fig. B).



Improve the expression of L-AAD with different plasmid copy number



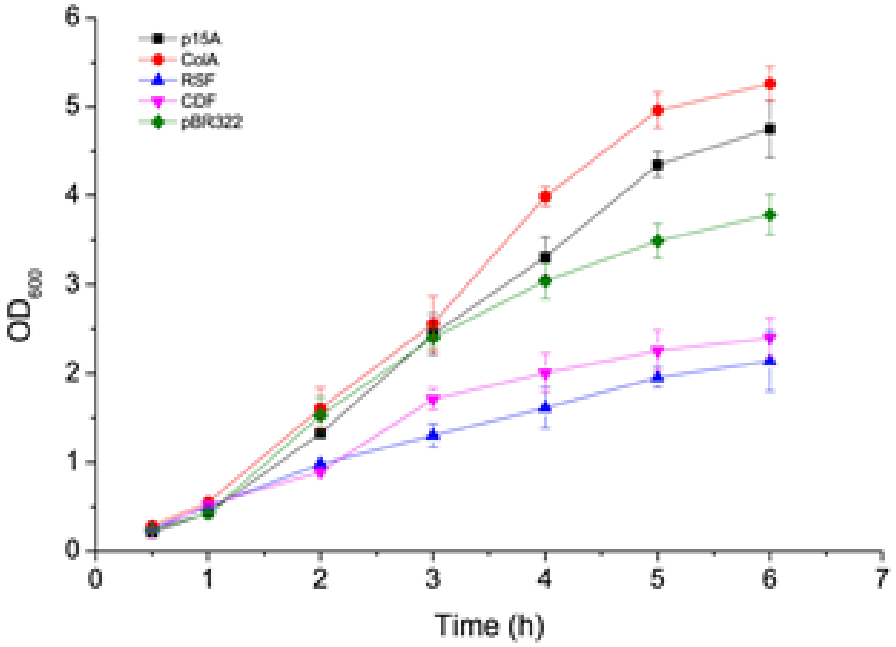
Different plasmid copy numbers

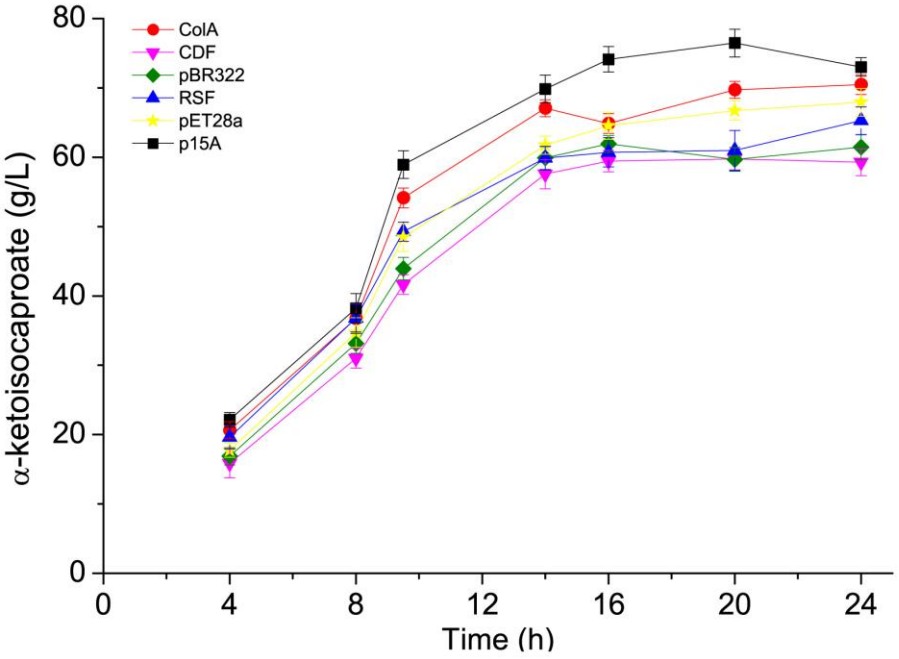
	p15A	ColA	RSF	CDF	pBR322
					
Copy number (a.u.)	10	5	100	20	40

a.u.: arbitrary units.

Improve the expression of L-AAD

- The effect of different plasmid copy number





- The cell growth rate increased with the decreased plasmid copy number.

- p15A (with 10 copy number) reached the highest KIC production 76.5 g/L when leucine was added at 2-h intervals (from 0 to 12 h).

Improve the expression of L-AAD

- **The effect of different plasmid copy number**

Comparison of different *ori* at RNA level, cell growth and KIC production

Plasmid	pACYCDuet-1	pColADuet-1	pCDFDuet-1	pRSFDuet-1	pETDuet-1
ori	p15A	ColA	CDF	RSF	pBR322
Copy number	10	5	20	100	40
Final OD_{600}	4.75	5.26	2.4	2.14	3.785
RNA level	1	-9.8	13.98	12.33	12.28
Production (g/L)	76.47	70.47	59.26	65.27	61.47
Biocatalyst activity (mg/g•min)	22.12	20.62	15.85	19.60	16.88

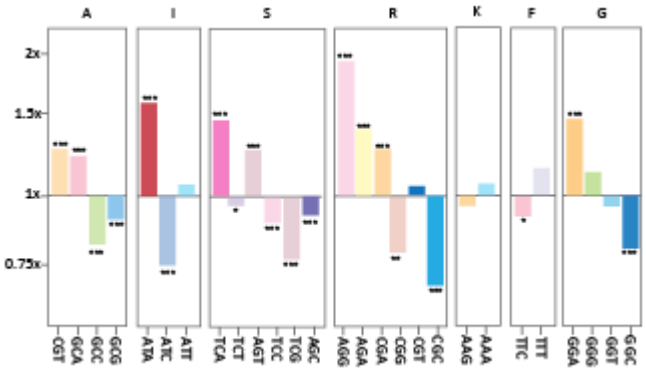
- **Higher plasmid copy number do not result in higher RNA level, which means that transcription of L-AAD is limited at higher plasmid copy number.**

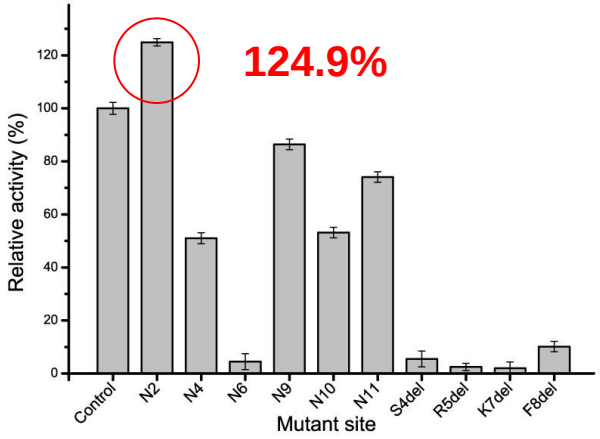
Improve the expression of L-AAD

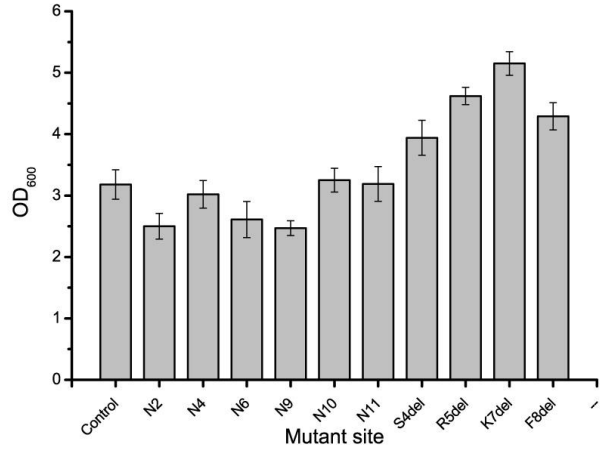
- **N-terminal codon bias and RNA structure affected KIC production**

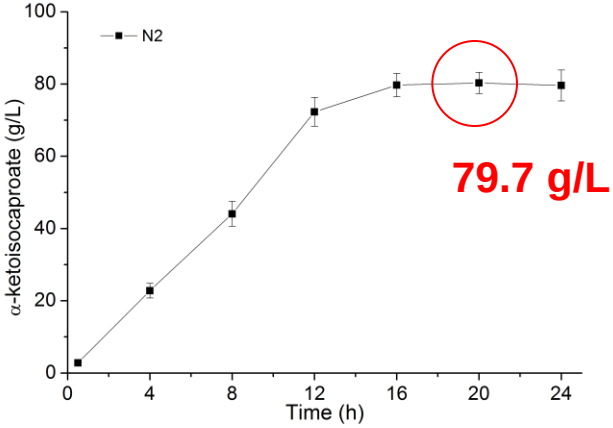
N-Terminal codons strongly related to ribosomal elongation. What' s more, N-terminal codons reduced mRNA secondary structure at the N terminus. So the mutants will change translational efficiency.

Mutants	ΔG (kcal/mol)
wild	-4.2
N2: CGT	-3.4
N4: TCA	-4.2
N5: AGG	-4.2
N6:AGG	-5.8
N9: ATA	-2.0
N10: ATA	-4.2
N11: GGA	-3.9
N4del	-5.3
N5del	-4.4
N3del	-2.2
N7del	-6.1
N8del	-2.20





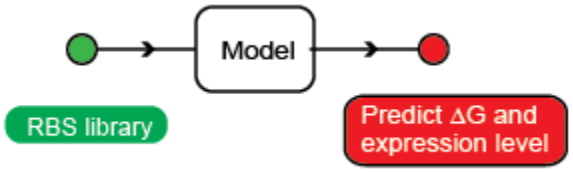


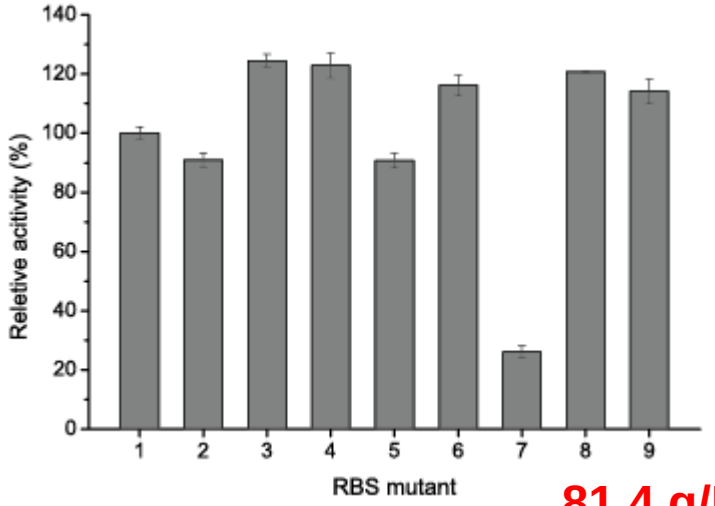


The highest KIC production reached 79.7 g/L

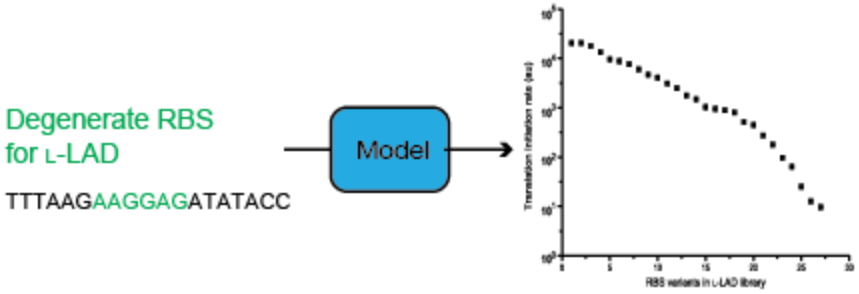
Improve the expression of L-AAD

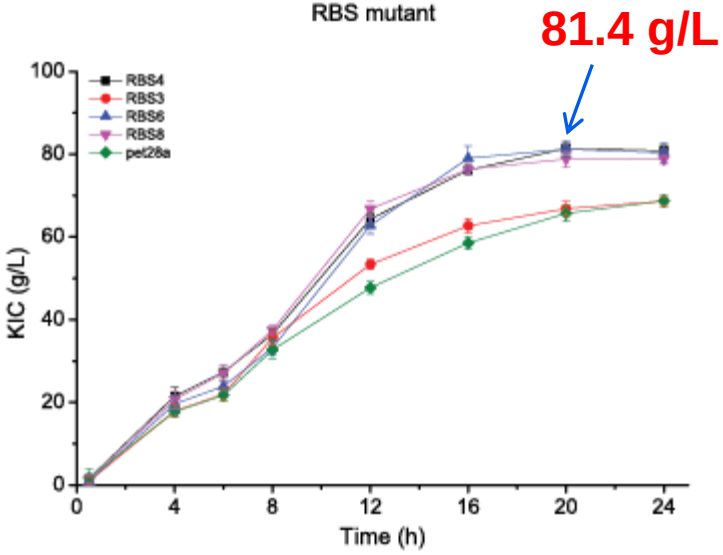
- Synthetic ribosome binding sites were optimized to control protein expression





By evaluating the degenerate RBS library and selecting synthetic RBS sequences with target ΔGs to improve the L-AAD expression level.





The highest KIC production reached 81.4 g/L

Summary

- **Biotransformation of L-glutamate to α-KG by L-AAD (pm1) from *P. mirabilis***

✓ N-terminus deleted L-AAD: the conversion rate is 12.6% and α-KG production is 1.5 g/L.

✓ Directed evolution, site-directed mutagenesis & gene shuffling of L-AAD and the deletion of product degradation pathway were performed, and the best mutant exhibited 67.7% of conversion rate and 58.6 g/L of α-KG in the fed-batch biotransformation system.

- **Biotransformation of L-phenylalanine to PPA by L-AAD (pma) from *P. mirabilis***

✓ Under the optimal conditions for 12 h, the maximal conversion rate and PPA titer reached 82.5% and 3.3 g/L, respectively.

✓ In 3-L fermentor, the conversion rate can be almost 100% within 4 h and no substrate inhibition was observed within 35 g/L of L-Phe.

- **Biotransformation of L-leucine to α-ketoisocaproate by L-AAD from *P. vulgaris***

✓ On the optimal conditions, the α-ketoisocaproate titer reached 12.7 g/L with a leucine conversion rate of 97.8%.

✓ The highest KIC production reached 76.5 g/L, 79.7 g/L, 81.4 g/L with the optimal the plasmid copy number, N-codon, RBS sequence, respectively.


Thanks for your attention!