Molecular Engineering of L-Amino Acid Deaminase and Optimization of Whole-Cell Catalysis Conditions for -Ketoglutaric Acid Production: Postprint

Authors: Wang Yue, Jianghua Li, Du Guocheng, Liu Long

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Abstract

-Ketoglutaric acid (-KG) is the keto acid product of glutamic acid deamination and, as an important organic acid, is widely used in fields such as food, medicine, and fine chemicals. To improve the efficiency and yield of -KG synthesis via whole-cell catalysis using L-amino acid deaminase, we first optimized the preparation conditions of the whole-cell catalyst and the whole-cell transformation reaction conditions, including temperature, pH, inducer concentration, timing of inducer addition, and induction time during the fermentation process; as well as temperature, pH, cell density, and transformation time during the whole-cell transformation process. After optimization of each condition, using 200 g · L ^{1} sodium glutamate as substrate, the final yield increased by 54.9% with a molar conversion rate of 39.6%. Secondly, directed evolution of L-amino acid deaminase was performed through site-directed saturation mutagenesis to enhance its catalytic capability. After multiple rounds of mutagenesis and screening, the optimal mutant E. coli BL21-pET-20b(+)-pm1152 catalyzed the production of -KG from 200 g · L ^{1} sodium glutamate with a maximum yield of 100.9 g · L ^{1} and a molar conversion rate of 64.7%, representing a 66.3% improvement over the initial control strain. The results demonstrate that condition optimization and saturation mutagenesis can effectively enhance the ability of recombinant Escherichia coli to synthesize -KG via whole-cell transformation.

Full Text

Molecular Modification of L-amino Acid Deaminase and Optimization of -ketoglutaric Acid Production by Whole-cell Biocatalysis

WANG Yue, LI Jiang-hua, DU Guo-cheng, LIU Long
(School of Biotechnology, Jiangnan University, Wuxi 214122, Jiangsu, China)

Abstract

-ketoglutaric acid (-KG) is the keto acid product of glutamic acid deamination and serves as an important organic acid widely used in food, pharmaceutical, and fine chemical industries. To improve the efficiency and yield of -KG synthesis via whole-cell biocatalysis using L-amino acid deaminase, we first optimized the preparation conditions of the whole-cell biocatalyst and the biotransformation reaction conditions. The optimization parameters included temperature, pH, inducer concentration, induction timing, and induction duration during the fermentation process, as well as temperature, pH, cell concentration, and reaction time during the whole-cell biotransformation. After optimizing each condition, the final yield increased by 54.9% with a molar conversion rate of 39.6% when using 200 g · L ^{1} sodium glutamate as substrate. Second, directed evolution of L-amino acid deaminase was performed through site-saturation mutagenesis to enhance its catalytic capacity. After multiple rounds of mutation and screening, the optimal mutant strain *E. coli* BL21-pET-20b(+)-pm1152 produced -KG at a maximum concentration of 100.9 g · L ^{1} from 200 g · L ^{1} sodium glutamate, achieving a molar conversion rate of 64.7%—a 66.3% improvement over the initial control strain. These results demonstrate that condition optimization and saturation mutagenesis can effectively enhance the ability of recombinant *E. coli* to synthesize -KG through whole-cell biotransformation.

Keywords: L-amino acid deaminase, condition optimization, site-saturation mutagenesis, whole-cell biocatalysis, CLC number: Q819

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Corresponding author: LIU Long, E-mail: longliu@jiangnan.edu.cn

Introduction

-ketoglutaric acid (-KG) is an important organic acid that serves as a key node connecting carbon and nitrogen metabolism, with widespread applications in food, pharmaceutical, and fine chemical industries [1-3]. Currently, -KG is primarily produced through chemical synthesis and fermentation methods. Chemical synthesis suffers from issues such as the use of toxic substances and low yield [4], while fermentation methods are limited by excessive byproducts and

long fermentation cycles [5], which restrict its application and industrial production. In previous studies, we have employed biotransformation methods to synthesize various keto acids including -ketoglutaric acid, ketoisocaproic acid, phenylpyruvic acid, and ketoisovaleric acid [6-10]. Whole-cell biotransformation offers multiple advantages: it provides a stable environment for enzymatic reactions and yields single products that are easy to separate [11]. L-amino acid deaminase (PM1) is a membrane protein that uses non-covalently bound flavin adenine dinucleotide (FAD) as a cofactor to catalyze the oxidative deamination of L-amino acids to produce corresponding -keto acids and ammonia [12-14]. In our previous work, we constructed an *E. coli* strain expressing pm1 that can catalyze the conversion of L-glutamic acid to -ketoglutaric acid [15]. This study employs two strategies to increase the production of -ketoglutaric acid (-KG) from sodium glutamate (MSG) substrate: first, optimizing whole-cell biocatalyst preparation and biotransformation conditions to enhance product synthesis, and second, performing directed evolution of PM1 through saturation mutagenesis to improve the enzyme' s catalytic capacity.

Materials and Methods

1.1 Materials

1.1.1 Reagents

Prime STAR HS DNA polymerase, 2×Taq PCR mix, bacterial competent cell preparation kit, and Blunting Kination Ligation (BKL) Kit 6127A were purchased from TAKARA (Dalian, China). Gene JET PCR kit was purchased from Thermo Fisher Scientific. San Prep column plasmid mini-prep kit, IPTG, and ampicillin were purchased from Sangon Biotech (Shanghai). -KG standard sample was purchased from Sigma-Aldrich.

1.1.2 Strains

E. coli BL21(DE3)-pET-20b(+)-pm1, constructed in our laboratory, is a recombinant *E. coli* BL21(DE3) overexpressing the pm1 gene with expression vector pET-20b(+).

1.1.3 Media

Seed culture medium was liquid LB: 10 g NaCl, 5 g yeast extract, and 10 g peptone per liter of water. Fermentation medium was TB: 5 g glycerol, 24 g yeast extract, 12 g peptone, 2.31 g KH PO , and 16.37 g K HPO ·3H O per liter of water. Solid LB medium: 10 g NaCl, 5 g yeast extract, 10 g peptone, and 20 g agar per liter of water.

1.2 Methods

1.2.1 Strain Activation and Culture

E. coli BL21(DE3)-pET-20b(+)-pm1 glycerol stock stored at -80°C was streaked onto solid LB medium containing ampicillin and incubated at 37°C overnight. A single colony was inoculated into liquid LB medium and cultured at 37°C

with shaking at 220 rpm. The culture was then transferred to TB medium at 2% inoculation volume. When the OD reached the appropriate value, IPTG was added at a certain concentration for induction.

1.2.2 Whole-cell Biocatalyst Preparation

After shake-flask cultivation, recombinant *E. coli* cells were harvested by centrifugation at 4°C and 5095×g for 10 min. The cell pellet was washed twice with buffer and resuspended in buffer to adjust the cell concentration to OD = 80-100, then stored at 4°C for later use. The conversion formula between dry cell weight (DCW, g · L ^{1}) and OD value is as follows:

$$\mathrm{DCW}(\mathrm{g}\cdot\mathrm{L}^{1}) = 0.4442 \times \mathrm{OD} \quad - 0.02 \qquad (1)$$

1.2.3 Optimization of Induction Temperature

Cultured seeds were inoculated at 2% volume fraction into 50 ml fermentation medium. Induction was performed at different temperatures (15°C, 20°C, 25°C, 30°C) when OD reached 2.0 by adding 0.04 mmol · L ^{1} IPTG. After 12 h, equal cell amounts were harvested to measure -KG production from 200 g · L ^{1} MSG substrate at 30°C.

1.2.4 Optimization of Induction Timing

Seed cultures were transferred to TB medium at 30-min intervals to obtain fermentation broths with different OD values. Broths with OD 2.0, 3.0, 4.0, and 5.0 were selected and induced with 0.04 mmol · L ^{1} IPTG simultaneously. After 12 h induction at 20°C, equal cell amounts were harvested to measure -KG production from 200 g · L ^{1} MSG substrate at 30°C.

1.2.5 Optimization of Induction Duration and Inducer Concentration

Cultured seeds were inoculated at 2% volume fraction into 50 ml fermentation medium. When OD reached 3.0, 0.04 mmol · L ^{1} IPTG was added for induction at 20°C. Cells were harvested after 8 h, 12 h, and 16 h to measure product formation from 200 g · L ^{1} substrate at 30°C. For inducer concentration optimization, IPTG concentrations of 0.01, 0.02, 0.04, 0.06, 0.1, 0.2, 0.4, and 0.8 mmol · L ^{1} were tested. Cultured seeds were inoculated at 2% volume fraction and grown until OD reached 3.0 before adding different IPTG concentrations. After 12 h induction at 20°C, equal cell amounts were harvested to measure -KG production from 200 g · L ^{1} substrate at 30°C.

1.2.6 Optimization of Seed Culture Time

Seed culture times of 8 h, 10 h, and 12 h were tested. In all cases, cultured seeds were inoculated at 2% volume fraction into 50 ml fermentation medium. When OD reached 3.0, 0.04 mmol · L ^{1} IPTG was added and induction continued at 20°C for 12 h. Equal cell amounts were then harvested to measure product formation from 200 g · L ^{1} substrate at 30°C.

1.2.7 Optimization of Reaction Temperature and pH

Reaction temperatures of 25°C, 30°C, 35°C, and 40°C were tested with DCW of 20 g · L ^{1} to measure product formation from 200 g · L ^{1} substrate. For pH optimization, phosphate buffer (pH 5.5-8.0) and Tris-HCl buffer (pH 7.5-9.0)

were prepared. Reactions were performed at 30°C with 20 g · L ¹ DCW and 200 g · L ¹ MSG for 48 h, after which -KG production was measured.

1.2.8 Optimization of Reaction Time and Cell Concentration

Equal cell amounts were used to measure product formation from 200 g · L ¹ substrate at 30°C at different reaction times. Cell concentrations (DCW) were varied from 5 g · L ¹ to 40 g · L ¹ in increments of 5 g · L ¹. Reactions were performed at 30°C with 200 g · L ¹ MSG, and -KG production was measured.

1.2.9 Construction of L-amino Acid Deaminase Structural Model and Substrate Docking

A crystal structure model of PM1 with 93.74% homology was obtained from the Swiss Model online server (PDB ID: 5FJM from *Proteus myxofaciens*) [16]. After homology modeling, AutoDock 4.0 software was used for molecular docking between the protein model and L-glutamic acid. Based on analysis of the active site key residues and interactions between the substrate, FAD, and enzyme, ten amino acid positions (I100, G206, G199, G235, P272, V276, V283, E340, L363, N406) were selected for single-site random mutagenesis.

1.2.10 Site-saturation Mutagenesis and Screening of L-amino Acid Deaminase

After determining mutation sites through software analysis, mutagenic primers were designed to randomly replace amino acid codons for library construction. The mutagenesis method involved whole-plasmid amplification followed by phosphorylation and ligation using the Blunting Kination Ligation (BKL) Kit. Primer names and sequences for site-saturation mutagenesis are shown in . For high-throughput screening, 100 random clones from the mutant library were picked into 96-well plates and cultured at 37°C for 10 h. Fifty percent of each culture was transferred to 48-well plates containing 0.04 mmol · L ¹ IPTG final concentration and induced at 37°C for 4 h, followed by centrifugation (4200×g, 5 min, 4°C) and supernatant removal. Substrate MSG at 50 g · L ¹ was dissolved in 0.2 M Tris-HCl buffer (pH 8.0). Six hundred microliters of substrate was added to each well containing cells for catalytic reaction at 37°C. After 1 h, the reaction was stopped by centrifugation, and -KG content in the supernatant was measured by colorimetric assay. The colorimetric method involved mixing 50 l supernatant with 100 l 0.1% 2,4-dinitrophenylhydrazine, incubating at room temperature for 5 min, adding 1 ml 1.5 M NaOH solution, and measuring absorbance at 520 nm after color stabilization. Mutants showing increased production from high-throughput screening were subjected to shake-flask re-screening to confirm improved catalytic efficiency.

1.2.12 -ketoglutaric Acid Quantification Method

-ketoglutaric acid concentration was measured by high-performance liquid chromatography (HPLC). Reaction samples (50 l) were diluted appropriately and centrifuged, and the supernatant was filtered through a 0.22 m membrane. The filtrate was used for HPLC analysis with the following conditions: Agilent 1260 HPLC system, column temperature 40°C, injection volume 10 l, mobile phase 5 mmol · L ¹ dilute H SO , UV detection wavelength 210 nm, flow rate 0.6 ml ·

min ^{1}, run time 15 min, Bio-Rad Aminex HPX-87H column (300 × 7.8 mm, 9 m).

Results and Discussion

2.1 Optimization of Fermentation Conditions

2.1.1 Optimal Induction Temperature

Induction was performed at four different temperatures (15°C, 20°C, 25°C, 30°C) when OD reached 2.0 with 0.04 mmol · L ^{1} IPTG. After 12 h, cells were harvested for whole-cell biotransformation of 200 g · L ^{1} MSG. As shown in [Figure 1: see original paper], the optimal induction temperature was 20°C, yielding the highest -KG concentration of 37.4 g · L ^{1} with a molar conversion rate of 23.9%.

2.1.2 Optimal Induction Timing

IPTG at 0.04 mmol · L ^{1} final concentration was added to cultures at different OD values (2.0, 3.0, 4.0, 5.0). After 12 h induction at 20°C, cells were harvested for whole-cell biotransformation of 200 g · L ^{1} MSG. As shown in [Figure 2: see original paper], the highest yield of 42.3 g · L ^{1} (27.1% molar conversion) was obtained when induction was performed at OD 2.0. However, the final OD value was lower under this condition, which was unfavorable for subsequent experiments. Induction at OD = 3.0 resulted in a higher final cell density with a comparable product yield of 41.4 g · L ^{1} (26.5% molar conversion). Therefore, OD = 3.0 was selected as the optimal induction timing.

2.1.3 Optimal Induction Duration and Inducer Concentration

Under 20°C induction conditions with OD 3.0 and 0.04 mmol · L ^{1} IPTG, cells were harvested after 8 h, 12 h, and 16 h for whole-cell biotransformation of 200 g · L ^{1} MSG. As shown in [Figure 3: see original paper], the maximum yield of 43.0 g · L ^{1} (27.5% molar conversion) was achieved after 12 h induction; longer induction times negatively affected product formation.

For inducer concentration optimization, different IPTG concentrations were tested at OD = 3.0 with 12 h induction at 20°C. As shown in [Figure 4: see original paper], product yield initially increased then decreased with increasing inducer concentration, reaching a maximum of 45.1 g · L ^{1} (28.9% molar conversion) at 0.06 mmol · L ^{1} IPTG. However, the cell density (OD) at 0.06 mmol · L ^{1} IPTG was significantly lower than at 0.04 mmol · L ^{1} IPTG, with similar product yields. Therefore, 0.04 mmol · L ^{1} IPTG was used for subsequent experiments.

2.1.4 Optimal Seed Culture Time

Based on the growth curve, seed cultures of different durations (8 h, 10 h, 12 h) were transferred to TB medium for induction. The resulting whole-cell biocatalysts were used for MSG biotransformation. As shown in [Figure 5: see original paper], the highest product yield of 42.2 g · L ^{1} (27.0% molar conversion) was obtained using 10 h seed culture.

Based on these results, the optimal fermentation conditions were: seed culture

time 10 h, inducer concentration 0.06 mmol · L ¹ IPTG, induction timing at OD 3.0, induction temperature 20°C, and induction duration 12 h.

2.2 Optimization of Biotransformation Conditions

2.2.1 Optimal Reaction Temperature and pH

Four temperature gradients (25°C, 30°C, 35°C, 40°C) were tested in Tris-HCl buffer (pH 8.0) with 20 g · L ¹ DCW and 200 g · L ¹ MSG. After 48 h reaction, -KG production was measured. As shown in [Figure 6: see original paper], the highest -KG yield of 42.0 g · L ¹ (26.7% molar conversion) was achieved at 30°C.

For pH optimization, phosphate buffer (pH 5.5-8.0) and Tris-HCl buffer (pH 7.5-9.0) were prepared. Reactions were performed at 30°C with 20 g · L ¹ DCW and 200 g · L ¹ MSG for 48 h. As shown in [Figure 7: see original paper], the maximum -KG yield of 54.9 g · L ¹ (35.2% molar conversion) was obtained in phosphate buffer at pH 6.0. Lower or higher pH values decreased catalytic efficiency, likely due to structural changes in the enzyme's active center reducing substrate binding efficiency. Phosphate buffer was superior to Tris-HCl buffer at the same pH, indicating it is more favorable for PM1-catalyzed conversion of MSG to -KG.

2.2.2 Optimal Reaction Time and Cell Concentration

In phosphate buffer (pH 6.0) at 30°C with 20 g · L ¹ DCW and 200 g · L ¹ MSG, the highest -KG yield of 61.9 g · L ¹ (39.6% molar conversion) was achieved after 64 h. Extended reaction time decreased yield, possibly because the rate of product formation became lower than the rate of cellular consumption as substrate concentration decreased [15].

Cell concentrations (DCW) from 5 g · L ¹ to 50 g · L ¹ were tested with 200 g · L ¹ MSG in phosphate buffer (pH 6.0) at 30°C. After 60 h, -KG production was measured. As shown in [Figure 9: see original paper], the maximum yield of 61.8 g · L ¹ (39.6% molar conversion) was achieved at 20 g · L ¹ DCW. Higher cell concentrations decreased yield, likely due to rapid product accumulation, reduced oxygen transfer rates at high cell densities, and the oxygen-consuming nature of the deaminase-catalyzed reaction [17].

In summary, the optimal biotransformation conditions were: reaction temperature 30°C, pH 6.0, reaction time 60 h, and cell concentration 20 g · L ¹ DCW.

2.3 Structural Modeling and Substrate Docking of L-amino Acid Deaminase

Molecular docking between L-amino acid deaminase and L-glutamic acid was performed using AutoDock 4.0 software. Based on crystal structure 5FJM [16], the FAD active site was identified as shown in [Figure 10: see original paper]-(a)(b). Amino acids near the FAD active site and substrate binding pocket ([Figure 10: see original paper]-(c)) were selected for site-saturation mutagenesis.

The interaction between substrate and enzyme is illustrated in [Figure 10: see original paper]-(d).

2.4 Site-saturation Mutagenesis of L-amino Acid Deaminase

After saturation mutagenesis of I100, G206, G199, G235, P272, V276, V283, E340, L363, and N406, mutants with significantly higher yields than the control were obtained. Sequencing identified the beneficial mutants as G206R, P272F, V276C, V283I, E340S, and E340G, which showed yield improvements of 12.5%, 18.2%, 14.8%, 4.5%, 27.6%, and 17.0%, respectively. The highest yield reached 77.9 g·L ^{1} with a molar conversion rate of 49.9% ([Figure 11: see original paper]).

Combinatorial mutagenesis of the single-point mutations yielded several multi-site mutants with improved performance, including P272L/V276C, V276C/V283I, V276C/E340S, V276C/E340G, P272L/V283I, P272L/E340S, P272L/E340G, V283I/E340S, P272L/V276C/V283I, P272L/V276C/E340G, V276C/V283I/E340G, P272L/V283I/E340S, P272L/V283I/E340G, P272L/V276C/V283I/E340S, G206R/P272L/V283I/E340G, and G206R/P272L/V276C/V283I/E340S ([Figure 12: see original paper]). These variants showed yield improvements of 27.3%, 28.5%, 42.2%, 25.8%, 5.4%, 23.7%, 22.1%, 16.5%, 16.1%, 28.6%, 34.7%, 35.1%, 30.3%, 53.6%, 49.2%, and 66.3%, respectively. The mutant G206R/P272L/V276C/V283I/E340S achieved the highest -KG yield of 100.9 g · L ^{1} with a molar conversion rate of 64.7%. This mutant was designated *E. coli* BL21-pET-20b(+)-pm1152.

2.5 Characterization of Modified L-amino Acid Deaminase

The mutant strain *E. coli* BL21-pET-20b(+)-pm1152 was cultivated under optimal conditions to obtain whole-cell biocatalyst. The effects of temperature, pH, and cell concentration on the biotransformation process were re-optimized in shake flasks to verify the properties of the engineered enzyme. As shown in [Figure 13: see original paper], the optimal temperature, pH, and cell concentration remained unchanged compared to the wild-type enzyme, indicating that the mutations did not alter the fundamental enzymatic properties.

Conclusion

Through optimization of recombinant *E. coli* whole-cell catalysis conditions, the optimal parameters were determined as: seed culture time 10 h, IPTG final concentration 0.06 mmol · L ^{1}, induction timing at OD 3.0, induction temperature 20°C, and induction duration 12 h. The optimal biotransformation conditions were: reaction temperature 30°C, pH 6.0, reaction time 60 h, and cell concentration 20 g · L ^{1} DCW. Under these conditions, 200 g · L ^{1} MSG substrate was converted to 61.8 g·L ^{1} -KG with a molar conversion rate of 39.6%. Furthermore, through site-saturation mutagenesis and multi-site combinatorial mutations, the mutant *E. coli* BL21-pET-20b(+)-pm1152 was obtained, which increased -KG production to 100.9 g · L ^{1}—a 66.3% improvement—with a molar

conversion rate of 64.7%. The mutations did not alter the enzyme's fundamental properties. This study provides a foundation for the industrial production of -KG.

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Note: Figure translations are in progress. See original paper for figures.