Engineering an Artificial Pathway to Improve the Bioconversion of Lysine into Chiral Amino Alcohol 2-Hydroxycadaverine Using a Semi-Rational Design

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Abstract: Amino alcohols are important compounds that are widely used in the polymer and pharmaceutical industry, particularly when used as chiral scaffolds in organic synthesis. The hydroxylation of polypeptide polymers may allow crosslinking between molecular chains through the esterification reactions of hydroxyl and carboxyl groups. Therefore, this may alter the functional properties of polypeptide polymers. 2-hydroxycadaverine (2HyC), as a new type of chiral amino alcohol, has potential applications in the pharmaceutical, chemical, and polymer industries. Currently, 2HyC production has only been realized via pure enzyme catalysis or two-stage whole-cell biocatalysis, which faces great challenges for scale-up production. However, the use of a cell factory is very promising for the production of 2HyC in industrial applications. Here, we designed and constructed a promising artificial pathway in Escherichia coli for producing 2HyC from biomass-derived lysine. This biosynthesis route expands the lysine catabolism pathway and employs two enzymes to sequentially convert lysine into 2HyC. However, the catalytic activity of wild-type pyridoxal phosphate-dependent decarboxylase from Chitinophaga pinensis (DC Cp) toward 3-hydroxylysine is lower, resulting in the lower production of 2HyC. Thus, the higher catalytic activity of DC Cp is desired for low-cost and expanded industrial applications of 2HyC. To improve the catalytic activity of DC Cp, a mutant library of DC Cp was first built using a semi-rational design. The Kcat/Km of mutant DC Cp (R53D/V94I) increased by 63%. A titer of 359 mg/L 2HyC was produced in shake flasks, with a 2HyC titer increase of 54% compared to control strain ML101. The results show that the production of 2HyC was effectively increased through a semi-rational design strategy. These findings lay the foundation for the development and utilization of renewable resources to produce 2HyC in microorganisms via an efficient, green, and sustainable biosynthetic strategy for further industrial application.

Keywords: chiral amino alcohol; hydroxylation; 2-Hydroxycadaverine; hydroxylysine; semi-rational design

1. Introduction

The core of synthetic biology is to reshape natural biological systems to produce natural or non-natural chemicals [1,2]. Recently many important high-value chemicals have been produced in microorganisms via synthetic biology, such as L-pipecolic acid [3], rosmarinic acid [4], pigments [5], caffeic acid [6], 5-hydroxyxctoine [7], quercetin [8], 2-keto-L-gulonic acid [9], ergothioneine [10], monoterpenes [11], ectoine [12], and glutarate [13].
These natural or non-natural chemicals and their derivatives are widely used in diverse fields [14].

Enzyme-mediated C-H hydroxylation is an attractive strategy for diversifying amino acids and their derivatives [15]. C-H hydroxylation is a promising strategy to synthesize hydroxy amino acids (HAAs), which are widely used in the chemical, feed, and medicinal industries [16]. For example, 5-hydroxytryptophan is the functional precursor for the synthesis of serotonin, exhibiting important pharmaceutical activity [17]. Moreover, N-hydroxy-pipecolic acid is an important systemically acquired resistance signal molecule [18]. Trans-4-hydroxy-L-pipecolic acid is a direct intermediate for the synthesis of bioactive cyclopeptide MBJ-0110 and bromopyrrole alkaloid damipipecolin [19].

The functionalization of amino acids and their derivatives has facilitated the production of compounds with multiple functions, thereby expanding their application fields and scope [20]. Hydroxylsines are a series of hydroxylated derivatives of lysine comprising 3-hydroxylsine, 4-hydroxylsine, 5-hydroxylsine, and dihydroxylsines, which are widely used as building blocks in the chemical, feed, and pharmaceutical industries [21]. Hydroxylsines are widely used as precursors in the pharmaceutical industry, such as palinavir [22], tambromycin [23], balanol, cepafungin I, and glidobactin A [24]. Hydroxylsines are also important monomers of polyamides, as their decarboxylation produces hydroxylated terminal diamines, which can be used to produce new bio-based polyamides [25].

Amino alcohols (AAs) are important adjuvants and ligands in organic synthesis and biology, with the dual chemical properties of amine and alcohol [26]. As shown in Figure 1B, bifunctional compounds of chiral AAs are widely applied in many important fields of medicine, fine chemicals, materials, and asymmetric catalysis [26]. AAs are the precursor of polypeptide drugs such as Octreotide, Ziconotide, and Alamethicin F-30, nucleotide drugs such as Glycopeptidolipid, quinolone drugs such as Levofloxacin and Prazufloxacin, and radioactive drugs such as Lopamidol [27]. In addition, AAs have low toxicity and low viscosity, inhibit corrosion, absorb carbon dioxide, and cure at room temperature. Zhang et al. reported the reductive amination of transaminase to synthesize 2-amino-2-phenylethanol, resulting in 68.6 g/L/d of volumetric productivity of 2-amino-2-phenylethanol [28]. Because biocatalysis has the advantage of high stereoselectivity, biocatalysis can provide a superior route for the synthesis of chiral AAs [29]. In particular, Steinreiber et al. reported the synthesis of chiral AA 2-amino-1-phenylethanol using the double enzyme cascade reaction of L-threonine aldolase and L-tyrosine decarboxylase [30].

2-hydroxycadaverine (2HyC) is a novel chiral AA with potential applications in medicine [31], chemistry, and materials [32]. 2HyC can be produced from L-lysine via hydroxylation and subsequent decarboxylation [21]. First, lysine was hydroxylated to generate 3-hydroxylsine by lysine hydroxylase. Subsequently, 3-hydroxylsine was decarboxylated by decarboxylase to form 2HyC [21,27]. Baud et al. reported the direct synthetic route of 2HyC from L-lysine via enzymatic reactions using purified lysine hydroxylase KDO1 and purified lysine decarboxylase [21]. However, pure enzyme catalysts are limited in their application due to their cumbersome purification steps and high cost [33]. Li et al. first established a two-stage biocatalytic approach for the direct production of 2HyC from L-lysine via whole-cell biotransformation [27].

In this work, an artificial route for the production of AA 2-hydroxycadaverine with lysine hydroxylase from Kineococcus radiotolerans (KDO) and pyridoxal phosphate-dependent decarboxylase from Chitinophage pinensis (DCp) overexpression was illustrated in Figure 1A. Firstly, lysine was hydroxylated to generate 3-hydroxylsine by lysine hydroxylase. Subsequently, 3-hydroxylsine was decarboxylated by DCp to form AA 2HyC. In the present study, a semi-rational design strategy was carried out to engineer DCp. DCp was generated using AlphaFold 2 [34], and molecular docking was performed using the AutoDock 4.26 package (The Scripps Research Institute, USA). A53, N54, V94, K95, and A97 were selected for site-directed saturation mutagenesis. With this method, a mutant DCp (R53D/V94I) was screened out with a 63% increase in catalytic efficiency relative to the
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The titer of 2HyC reached 359 mg/L in 24 h. Moreover, mutants of DC<sub>CP</sub> with higher catalytic efficiency toward 3-hydroxylysine were obtained using the semi-rational design, which provides a theoretical foundation and preliminary information for the production of 2HyC to meet industrial requirements.

Figure 1. (A) The heterogeneous pathway for 2HyC production from lysine in *E. coli*. 2HyC, 2-hydroxy cadaverine. (B) Bifunctional compounds and chiral amino alcohols are widely applied in many important fields. Red “×” represents gene deletion.

2. Materials and Methods

2.1. Strains and Plasmids

The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* BL21(DE3) was used as the host strain to produce 2-hydroxy cadaverine. Strain BL21(DE3) with the *cadA* gene knocked out was conducted in our previous studies [3]. The nucleotide sequence of the lysine hydroxylase (EC 1.14.11.4) gene from *Kineococcus radiotolerans*...
(KDO\textsubscript{Kr}) is available in GenBank with the accession number ABS05421.1. The protein sequence of pyridoxal phosphate-dependent decarboxylase (EC 4.1.1) from \textit{Chitinophaga pinensis} (DC\textsubscript{Cp}) is available in GenBank with the accession number WP_012790490.1. Codon-optimized KDO\textsubscript{Kr} (Seen in Supplementary Data S1) was chemically synthesized and inserted into pET22b to form plasmid pET22b-KDO\textsubscript{Kr} with NdeI and BamHI restriction sites. Codon-optimized DC\textsubscript{Cp} (as seen in Supplementary Data S2) was chemically synthesized and inserted into pET22b and pET22b-KDO\textsubscript{Kr} to form plasmid pET22b-DC\textsubscript{Cp} and pET22b-KDO\textsubscript{Kr}-DC\textsubscript{Cp} with SalI and XhoI restriction sites, respectively. The sequences of all vector constructs were verified using Sanger sequencing (Sangon Biotech Co., Ltd., Shanghai, China).

**Table 1. The strains and plasmids used in this study.**

<table>
<thead>
<tr>
<th>Strains or Plasmids</th>
<th>Relevant Genotype or Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Wild type</td>
<td>[35]</td>
</tr>
<tr>
<td>ML03</td>
<td>BL21(DE3) ΔcadA</td>
<td>[3]</td>
</tr>
<tr>
<td>ML10</td>
<td>ML03 harboring pET22b-KDO\textsubscript{Kr}</td>
<td>This study</td>
</tr>
<tr>
<td>ML101</td>
<td>ML03 harboring pET22b-KDO\textsubscript{Kr}-DC\textsubscript{Cp}</td>
<td>This study</td>
</tr>
<tr>
<td>ML102</td>
<td>ML03 harboring pET22b-KDO\textsubscript{Kr}-DC\textsubscript{Cp} (R53D/V94I)</td>
<td>This study</td>
</tr>
<tr>
<td>ML11</td>
<td>ML03 harboring pET22b-DC\textsubscript{Cp}</td>
<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pET22b-KDO\textsubscript{Kr}</td>
<td>pET22b carries a lysine hydroxylase gene from \textit{Kineococcus radiotolerans} (KDO\textsubscript{Kr}), Amp\textsuperscript{r}</td>
<td>This study</td>
</tr>
<tr>
<td>pET22b-DC\textsubscript{Cp}</td>
<td>pET22b carries a pyridoxal phosphate-dependent decarboxylase gene from \textit{Chitinophaga pinensis} (DC\textsubscript{Cp}), Amp\textsuperscript{r}</td>
<td>This study</td>
</tr>
<tr>
<td>pET22b-KDO\textsubscript{Kr}-DC\textsubscript{Cp}</td>
<td>pET22b carries a lysine hydroxylase gene from \textit{Kineococcus radiotolerans} (KDO\textsubscript{Kr}), and a pyridoxal phosphate-dependent decarboxylase gene from \textit{Chitinophaga pinensis} (DC\textsubscript{Cp}), Amp\textsuperscript{r}</td>
<td>This study</td>
</tr>
<tr>
<td>pET22b-KDO\textsubscript{Kr}-DC\textsubscript{Cp} (R53D/V94I)</td>
<td>pET22b carries a lysine hydroxylase from \textit{Kineococcus radiotolerans} (KDO\textsubscript{Kr}), and a pyridoxal phosphate-dependent decarboxylase (DC\textsubscript{Cp}) mutant (R53D/V94I) from \textit{Chitinophaga pinensis} (DC\textsubscript{Cp}), Amp\textsuperscript{r}</td>
<td>This study</td>
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2.2. Culture Medium and Conditions

\textit{E. coli} ML10, ML101, or ML11 strains were cultured in 100 mL flasks containing 10 mL of the Luria–Bertani (LB) medium supplemented with 100 µg/mL of Amp for 12 h at 37 °C and 220 rpm. Then, 20 µL of the culture was transferred into a 100 mL flask containing 20 mL of the medium (comprising 10 g/L of tryptone, 5 g/L of yeast extract, 0.1 mM of pyridoxal phosphate, 0.5 g/L of K\textsubscript{2}H\textsubscript{PO}_{4}, 3 g/L of KH\textsubscript{2}PO\textsubscript{4}, 0.75 g/L of FeSO\textsubscript{4}·7H\textsubscript{2}O, 15 g/L of glucose, 20 of mM \textalpha{-}ketoglutarate and 100 µg/mL of Amp) at 37 °C and 220 rpm. After the OD\textsubscript{600} reached 0.6, 0.5 mM IPTG and 4 g/L lysine was added and continued in the culture at 30 °C. L-lysine, 3-hydroxylysine, and 2-hydroxycaverine were measured using HPLC.

The production of 2HyC in recombinant \textit{E. coli} ML101 and ML102 was conducted in 250 mL flasks. Strains ML101 and ML102 from the glycerol stock were streaked onto LB agar plates supplemented with 100 µg/mL Amp and grown overnight in an incubator at 37 °C. Three colonies were selected from an agar plate and grown in 5 mL of LB medium supplemented with 100 µg/mL Amp in an orbital shaker operated overnight at 220 rpm and 37 °C. The culture was adopted as the seed inoculum to initiate fermentation in triplicate. The fermentation medium was inoculated with the seed broth at 1% dilution and grown in 40 mL of the LB medium (comprising 10 g/L of tryptone, 5 g/L of yeast extract, 0.1 mM of pyridoxal phosphate, 0.5 g/L of K\textsubscript{2}H\textsubscript{PO}_{4}, 3 g/L of KH\textsubscript{2}PO\textsubscript{4}, 0.75 g/L of FeSO\textsubscript{4}·7H\textsubscript{2}O, 15 g/L of glucose, 20 mM of \textalpha{-}ketoglutarate and 100 µg/mL of Amp). The
initial pH was adjusted to 7.0. After the OD_600 reached 0.6, 4 g/L of L-lysine was added to the fermentation medium as a substrate at this time, and IPTG was added to a final concentration of 0.5 mM to induce the protein expression. The flasks were placed in an orbital shaker operated at 220 RPM and 30 °C. Experiments were performed in triplicate, and samples were taken at 12 and 24 h for 2HyC analysis.

2.3. Protein Expression and Purification

For the protein expression, ML03 harboring pET22b-DC_{Cp} was screened on selective LB agar plates supplemented with 100 µg/mL of Amp. Positive clones were inoculated in 2 mL of the fresh LB medium at 37 °C and 220 rpm for 12 h. A total of 2 mL of seed cultures was transferred into 200 mL of LB containing 100 µg/mL of Amp. When the OD_600 reached 0.6, a final concentration of 0.5 mM IPTG was employed and incubated for a low-temperature induction culture at 20 °C for 16 h. The cells were collected via centrifugation at 10,000 RPM for 5 min at 4 °C. The cells were resuspended and washed twice with a potassium phosphate buffer (KPB, 50 mM, pH 8.0), and 2 mM of tris (2-carboxyethyl) phosphine (TCEP) was added in an ice bath. The cells were disrupted via ultrasonication for 15 min in an ice bath. The enzymes were purified using an AKTA Purifier 10 and a Ni-NTA column (GE Healthcare, Chicago, IL, USA). The purified enzymes were desalted and exchanged into a storage buffer (50 mM of KPB, 1.0 mM of MgSO_4, 2 mM of TCEP, 10% glycerol, and pH 8.0) and stored at −80 °C [36]. The UV absorbance at 280 nm was used to measure the protein concentration using SpectraMax M2e (Molecular Devices, San Jose, CA, USA) [37,38].

2.4. Homology Modeling and Molecular Docking

The theoretical structures of native KDO_\beta and DC_{Cp} were generated using AlphaFold2 [34]. The initial structure was processed with AutoDockTools 1.5.6, preserving the original charge of the protein and generating a pdbqt file for docking. The ligands L-lysine and 3-hydroxylysine were docked into the pocket of KDO_\beta and DC_{Cp} using the AutoDock 4.2.6 package, respectively, where the lowest energy conformation in the largest cluster was considered to be an approximately natural complex model [39]. The energy optimization used the Amber14 force field.

2.5. Site-Directed Saturation Mutagenesis

*E. coli* was used as the chassis cell to screen DC_{Cp} mutants via an activity-linked screening method. For the construction of DC_{Cp} mutants, a semi-rational design strategy based on structural orientation was used. Here, the structure was modeled by AlphaFold2 and docked with 3-hydroxylysine. First, saturation mutagenesis was conducted at potential improvement sites in DC_{Cp} (accession number: WP_012790490.1) through whole plasmid PCR using degenerate primers. Degenerate primers containing NNK (the forward primer) were used for saturation mutagenesis at the corresponding mutation sites, where N represents the bases T, A, C, or G, and K represents T or G. To eliminate the primary template, the PCR products were digested by DpnI at 37 °C for 1 h and then directly transformed into *E. coli* BL21(DE3) competent cells to produce the variants. All the positive mutations were transformed into *E. coli* BL21(DE3) for the expression of the DC_{Cp} protein. The DC_{Cp} activity and protein concentration were measured to calculate the increase in DC_{Cp} activity for screened mutants. All experiments were carried out in triplicate.

2.6. Enzyme Assay

The determination of DC_{Cp} activity was carried out according to the method described by Prell et al. [40]. In total, 1.5 mL of the reaction mix contained 50 mM of the HEPES buffer (pH 7.5), 1 mM of PLP, 1 mM of DTT, and 10 mM of 3-hydroxylysine [21,40].
The reaction was started by the addition of 1 mg/mL of the protein. The reaction was conducted at 30 °C and stopped by adding 10 µL of 10 M HCl. All experiments were carried out in triplicate.

2.7. Analytical Methods

L-lysine, 3-hydroxylysine, and 2-hydroxycadaverine were analyzed and quantitated using HPLC (Agilent Technologies 1200 series, Hewlett-Packard, Palo Alto, CA, USA). The sample was derivatized with phenyl isothiocyanate (PITC) for the detection of lysine [18]. Samples were analyzed using HPLC with a C18 column (4.6 × 250 mm) and a Chirex®3126 (D)-penicillamine LC column (4.6 × 250 mm, Phenomenex, Torrance, CA, USA) as described by Cheng et al. [3]. The samples were centrifuged and filtered using a 0.22 µm membrane.

3. Results and Discussion

3.1. Construction of a Heterogeneous Pathway for 2HyC Production in Engineered E. coli ML101

To engineer the 2HyC pathway, pET22b-KDO<sub>Kr</sub>-DC<sub>Cp</sub> was constructed for the synthesis of 2HyC (Figure 1A). The designed heterogeneous pathway of 2HyC consists of two steps. The first step was to convert lysine into 3-hydroxylysine mediated by KDO<sub>Kr</sub>. The second step was to convert 3-hydroxylysine into 2HyC mediated by DC<sub>Cp</sub>. In this study, we tried to introduce a decarboxylase from Chitinophage pinensis (DC<sub>Cp</sub>) in combination with KDO<sub>Kr</sub> for 2HyC production from lysine. The sequence of KDO<sub>Kr</sub> and DC<sub>Cp</sub> was optimized according to the codon preference of E. coli. Firstly, plasmid pET22b-KDO<sub>Kr</sub>-DC<sub>Cp</sub> was constructed. Then, the plasmid pET22b-KDO<sub>Kr</sub>-DC<sub>Cp</sub> was transformed into E. coli ML103 and strain ML101 was obtained. As shown in Table 2, we investigated the production of 3-hydroxylysine and 2HyC from lysine in strain ML101. When 2 g/L of L-lysine was fed to recombinant E. coli ML101, 106.18 ± 5.48 mg/L of 2HyC was obtained after 12 h (Table 2). A titer of 195.37 ± 8.95 mg/L of 2HyC was produced with 4 g/L of lysine added after 12 h. Our data clearly demonstrate that the heterogeneous pathway of 2HyC in E. coli is feasible. Although the 2HyC synthesis pathway we have established can synthesize 2HyC, the titer is relatively low. We hypothesized that the low enzymatic activity of DC<sub>Cp</sub> was the main limitation [21].

### Table 2. Production of 3-hydroxylysine and 2HyC from lysine via recombinant E. coli ML10 and ML101.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Time (h)</th>
<th>2 g/L Lysine</th>
<th>4 g/L Lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3-Hydroxylysine Production (g/L)</td>
<td>2HyC Production (mg/L)</td>
</tr>
<tr>
<td>ML10</td>
<td>12</td>
<td>0.64 ± 0.05</td>
<td>-</td>
</tr>
<tr>
<td>ML101</td>
<td>12</td>
<td>0.47 ± 0.05</td>
<td>106.18 ± 5.48</td>
</tr>
</tbody>
</table>

3.2. Molecular Docking Studies

To gain a better insight into the interaction between KDO<sub>Kr</sub> and L-lysine on the molecular level, a molecular docking study was performed. As shown in Figure 2A, after 108 accurate runs, the mainly 12 binding sites on the KDO<sub>Kr</sub> were observed with the binding energy of ~4.95 kcal/mol for the strongest binding site. According to the principle of minimum energy, the conformation with the lowest binding energy was selected as the final result of molecular docking. As shown in Figure 2B, L-lysine was mainly bound to KDO<sub>Kr</sub> in the hydrophobic cavity, forming a hydrophobic interaction with the surrounding amino acids under the molecular docking binding energy of ~4.95 kcal/mol.
Figure 2. Structural modeling of KDO\(_{Kr}\) and molecular docking. (A) Overall architecture of KDO\(_{Kr}\); (B) Interactions of the ligand L-lysine with their surroundings in the KDO\(_{Kr}\) system; the cartoon model represents the KDO\(_{Kr}\) structure; (C) 3D interaction diagram of L-lysine with KDO\(_{Kr}\) protein. (D) Schematic diagram of the interactions of KDO\(_{Kr}\) with L-lysine (a green dotted line indicates hydrogen bond and a red dotted line indicates a hydrophobic interaction). KDO\(_{Kr}\), lysine hydroxylase, from *Kineococcus radiotolerans*.

After that, the analysis of the binding mode was performed to specifically clarify the binding mechanism. As shown in Figure 2C,D, the docking results of KDO\(_{Kr}\) and L-lysine showed that L-lysine formed four hydrogen bonds with the side chains E123, G124, S144, and R305. In addition, the binding of KDO\(_{Kr}\) with L-lysine could be attributed to a hydrophobic interaction because strong hydrophobic interactions of L-lysine with N93, M132, Q143, and Q145 were formed. The molecular docking results showed that L-lysine could bind to the hydrophobic cavity of KDO\(_{Kr}\) due to the formation of hydrogen bonds and hydrophobic interactions with surrounding amino acid residues.

In order to analyze the interaction between 3-hydroxylysine and DC\(_{Cp}\), we analyzed the interaction between 3-hydroxylysine and DC\(_{Cp}\) at the best binding site, as shown in Figure 3. As seen in Figure 3A,B, it can be observed that 3-hydroxylysine can stably bind to the active cavity formed by the interweaving of several helical structures in DC\(_{Cp}\). Furthermore, the analysis of the interaction between 3-hydroxylysine and DC\(_{Cp}\) shows that there are a large number of hydrogen bond donors and receptor groups in 3-hydroxylysine, which forms a large number of hydrogen bonds with amino acids around the pocket to promote the stable binding of 3-hydroxylysine to DC\(_{Cp}\). As seen in Figure 3C,D, 3-hydroxylysine forms hydrogen bonds with five amino acid residues around the DC\(_{Cp}\) pocket. The docking results of DC\(_{Cp}\) and 3-hydroxylysine showed that 3-hydroxylysine formed
five hydrogen bonds with the side chain A53, N54, V94, K95, and A97. In addition, a strong hydrophobic interaction of 3-hydroxylysine with K95 was formed, further enhancing the affinity between 3-hydroxylysine and DC_{Cp}.

Figure 3. The binding pattern diagram of 3-hydroxylysine with the DC_{Cp} protein. (A) The distribution of 3-hydroxylysine on the DC_{Cp} protein surface; (B) The spatial position of 3-hydroxylysine in the DC_{Cp} protein; (C) the 2D interaction diagram of 3-hydroxylysine with the DC_{Cp} protein. The green dashed line is for hydrogen bonding, and the red dotted line indicates hydrogen bond; (D) 3D interaction diagram of 3-hydroxylysine with the DC_{Cp} protein. DC_{Cp}, pyridoxal phosphate-dependent decarboxylase from Chitinophaga pinensis. The red circle represents oxygen atoms, the blue circle represents nitrogen atoms, and the black circle represents carbon atoms.

3.3. Iterative Saturation Mutagenesis of DC_{Cp} to Improve the Catalytic Activity toward 3-Hydroxylysine

Although we constructed an artificial biosynthetic route of chiral amino alcohol 2HyC when we introduced KDO_{Kr} and DC_{Cp} into E. coli ML03 (ML101) and cultivated ML101 in a flask, the accumulation of 2HyC in the culture was very low. In order to promote the production of 2HyC, we hoped to improve the catalytic capability of DC_{Cp}. To enhance the production of 2HyC, a mutant library of DC_{Cp} was established using a semi-rational design to improve the catalytic activity of DC_{Cp}. The semi-rational design of enzymes based on the 3D structure and molecular docking has been proven to reduce the workload of constructing and screening mutation libraries [41,42]. Therefore, sites R53,
N54, V94, K95, and A97 of DC\textsubscript{Cp} were selected for subsequent iterative saturation mutagenesis.

The enzyme assays showed that the saturation mutants of N54, K95, and A97 could not enhance the specific activity. Most of the substitutions at R53 and V94 reduced the specific activity, and two mutants had no significant effect on the specific activity. The specific activity of mutations R53D, R53E, V94A, and V94I was significantly higher than wild type (Table 3). DC\textsubscript{Cp} exhibited low activity toward 3-hydroxylysine, whereas DC\textsubscript{Cp} mutations (R53D/V94I and R53E/V94I) displayed enhanced activities in Table 3. The DC\textsubscript{Cp} variant R53D/V94I showed the greatest activity in Table 3. Wild-type DC\textsubscript{Cp} showed a $K_m$ value of 20.72 mM, a $K_{cat}$ value of 1.86 s$^{-1}$, and a $K_{cat}/K_m$ value of 0.0898 mM$^{-1}$s$^{-1}$ when 3-hydroxylysine was used as the substrate. The $K_{cat}/K_m$ of the mutant DC\textsubscript{Cp} (R53D/V94I) increased by 0.63-fold compared to wild-type DC\textsubscript{Cp}. These findings promote an understanding of the structure–function relationship of DC\textsubscript{Cp} and increase its catalytic efficiency for further industrial application. The enzyme activity assay showed that the DC\textsubscript{Cp} enzyme has no activity against lysine.

Table 3. Kinetic parameters of DC\textsubscript{Cp} and its mutant on 3-hydroxylysine.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$K_m$ (mM)</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
<th>$K_{cat}/K_m$ (mM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC\textsubscript{Cp}</td>
<td>20.72 ± 1.24</td>
<td>1.86 ± 0.12</td>
<td>0.0898 ± 0.0035</td>
</tr>
<tr>
<td>DC\textsubscript{Cp} (R53D)</td>
<td>18.05 ± 1.02</td>
<td>2.08 ± 0.15</td>
<td>0.1152 ± 0.0058</td>
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<tr>
<td>DC\textsubscript{Cp} (R53E)</td>
<td>19.21 ± 0.89</td>
<td>2.13 ± 0.18</td>
<td>0.1109 ± 0.0043</td>
</tr>
<tr>
<td>DC\textsubscript{Cp} (V94A)</td>
<td>18.48 ± 1.07</td>
<td>2.24 ± 0.16</td>
<td>0.1212 ± 0.0066</td>
</tr>
<tr>
<td>DC\textsubscript{Cp} (V94I)</td>
<td>17.96 ± 1.35</td>
<td>2.19 ± 0.13</td>
<td>0.1097 ± 0.0074</td>
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<tr>
<td>DC\textsubscript{Cp} (R53E/V94I)</td>
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<td>2.25 ± 0.19</td>
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<tr>
<td>DC\textsubscript{Cp} (R53D/V94I)</td>
<td>16.33 ± 1.18</td>
<td>2.39 ± 0.17</td>
<td>0.1466 ± 0.0067</td>
</tr>
</tbody>
</table>

Data are presented as the means ± STDV calculated from at least three replicates. The determination of DC\textsubscript{Cp} activity was conducted on a 1.5 mL reaction mix containing 50 mM of the HEPES buffer (pH 7.5), 1 mM of PLP, 1 mM of DTT and 10 mM of 3-hydroxylysine. The reaction was started by the addition of 1 mg/mL of the protein. The reaction was conducted at 30 °C and stopped by adding 10 µL of 10 M HCl.

Currently, there is no study on enhancing 2HyC production using enzyme engineering strategies or semi-rational design strategies. Previous studies usually use metabolic engineering to enhance 2HyC production, including the heterologous expression of key genes and using a purified enzyme catalytic system [21]. Baud et al. reported a direct synthetic route of 2HyC from L-lysine via enzymatic reactions using purified lysine hydroxylase KDO1 and purified lysine decarboxylase [21]. However, pure enzyme catalysts are limited in their application due to their cumbersome purification steps and high cost [33]. Recently, a two-stage biocatalytic approach for the direct production of 2HyC from L-lysine via whole-cell biotransformation was first established by Li et al. [27]. In the decarboxylation step, decarboxylase CadA can decarboxylate not only 3-hydroxylysine but also lysine [27]. Therefore, 2HyC can only be synthesized using a two-stage biocatalytic approach in this research [27]. The enzyme engineering strategy can include the iterative saturation mutagenesis of the key sites to improve the activity of the enzyme. In the present study, DC\textsubscript{Cp} (R53D/V94I) produced the highest titer of 2HyC. V94 is located in the substrate pocket, so V94 may change the shape of the hydrophobic cavity of the substrate pocket.
3.4. Metabolic Engineering of E. coli ML102 for 2HyC Production

2HyC is accessible as the product of the direct hydroxylation and decarboxylation of lysine; 2HyC, on the other hand, is a non-naturally occurring compound; thus, it is not described in organisms naturally. Based on the enzyme catalytic properties measured in vitro, we sought to build this artificial metabolic route in E. coli to produce 2HyC by expressing KDOKr and DC_{Cp} enzymes, as seen in Figure 1A. In order to improve the catalytic capability of DC_{Cp}, a mutant library of DC_{Cp} was established through a semi-rational design strategy. The greatest activity of the DC_{Cp} variant R53D/V94I was obtained. To further evaluate the potential fermentation performance of the DC_{Cp} variant R53D/V94I, fermentation evaluation was conducted on strain ML102. After 24 h of aerobic cultivation, there was no accumulation of 2HyC in the control strain ML03. The OD_{600} reached 4.05 after 8 h and 5.86 after 12 h. On the other hand, the engineered strain ML101 and ML102 produced 2HyC with a peak concentration of 195.37 mg/L and 304.27 mg/L at 12 h, as shown in Figure 4, respectively. The successful assembly of this artificial pathway in E. coli validates the feasibility of producing L-lysine-derived 2HyC. In this study, our engineered E. coli strain ML102 could use lysine as a substrate to produce 2HyC with a titer of 359 mg/L after 24 h, with a 2HyC titer increase of 54% compared to the control strain ML101, as seen in Figure 4.

![Figure 4](image-url) An artificial pathway confirmed for the biosynthesis of 2HyC via E. coli ML101 and ML102 in 250 mL flasks. A total of 4.0 g/L of L-Lysine was used as a substrate. Values and error bars represent the mean and the standard deviation of triplicate.

4. Conclusions

Chiral AAs have tremendous utility in various industries, including adjuvants and ligands. In conclusion, we described a biotransformation system utilizing KDOKr and DC_{Cp} to convert L-lysine to 2HyC in E. coli. An engineered E. coli strain, ML102, with KDOKr and DC_{Cp} (R53D/V94I) overexpression, can produce 359 mg/L of 2HyC from L-lysine in a 250 mL flask. This work provides a sustainable route for industrial 2HyC production from renewable feedstocks using metabolic engineering. This study started with the structure prediction of DC_{Cp}. Subsequently, the double mutant DC_{Cp} (R53D/V94I) was obtained via iterative saturation mutagenesis. In this study, DC_{Cp} mutants with catalytic activity enhanced up to 0.63-fold were obtained from a smart mutant library using a semi-rational design. These findings promoted an understanding of the structure–function relationship of DC_{Cp} and improved its catalytic efficiency for industrial application. This study demonstrates the production of 2HyC in E. coli, which is an important step in developing E. coli
into an industrial strain for producing 2HyC. Future engineering efforts can utilize directed evolution and rational design strategies to produce a higher titer of 2HyC in E. coli, although additional enzyme and strain optimization may be required.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/10.3390/fermentation10010056/s1, Table S1: The OD900 value at specific times of strains ML101 and ML102; Data S1: The codon-optimized nucleotide sequence of lysine hydroxylase from Kineococcus radiotolerans (KDOx); Data S2: The codon-optimized nucleotide sequence of pyridoxal phosphate-dependent decarboxylase from Chitinophaga pinensis (DC0).

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References


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