An efficient procedure for the production of *trans*-4-hydroxy-L-proline using recombinantly expressed proline hydroxylase

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Abstract. Due to the codon usage and high G+C content of the *trans*-4-proline-L-hydroxylase gene from the *Dactylosporangium sp.* strain RH1, the whole gene was optimized and cloned into several vectors for expression. In biotransformations with resting cells, the activity of the enzyme was investigated. The in-house modified plasmid pET-M-3C was found to yield the highest enzymatic activity. Additionally, after the primary fragment screening, the conversion efficiency of fragment 1-257 aa was enhanced from 76.60% to 88.97% compared with the full-length proline 4-hydroxylase within 60 h; we also found that truncation of the gene improved the solubility of the encoded protein. After optimizing various induction conditions with respect to the enzymatic activity of the engineered strain, the conversion efficiency was more than 97% within 48 h.

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1. Introduction

Enzymatic catalysis has become increasingly important in industrial production. Compared to chemical synthesis, enzyme catalysis has the advantages of being environmentally friendly and inexpensive. Proline hydroxylases are an important group of enzymes that can hydroxylate free L-proline to hydroxy-L-proline with high regio- and stereo-selectivity. Proline hydroxylases can be classified into four groups based on their products: *cis*-3-, *cis*-4-, *trans*-3-, and *trans*-4-proline hydroxylase [1-5].

Prolyl 4-hydroxylase belongs to a family of α-oxoglutarate-dependent dioxygenases that require α-oxoglutarate and non-heme ferrous iron. Prolyl 4-hydroxylase can hydroxylate peptidyl L-proline to peptidyl hydroxy-L-proline [6,7] but not to free L-proline. Katz et al. (1979) reported that proline 4-hydroxylase belongs to a family of α-oxoglutarate-dependent dioxygenases and prolyl hydroxylases that are involved in collagen biosynthesis [8]. *Trans*-4-proline hydroxylase (HYD) can hydroxylate free L-proline to *trans*-4-hydroxy-L-proline, which is a useful compound for the chemical synthesis of certain pharmaceuticals, such as carbapenem antibiotics, angiotensin-converting enzyme inhibitors, antispastic agents, and antiphlogistics [9].

HYD activity has been observed during the biosynthesis of etamycin in *S. griseoviridus* P-8648, and high activity levels of HYD have been identified

in *Streptomyces sp.* strain RH1. HYD has also been cloned and expressed in *E. coli* [5,10-11].

Klein et al. (2011) reported that several commercial vectors yielded a protein that was almost insoluble in *E. coli*, and a reasonable fraction of soluble and active protein was obtained through coexpression with the chaperone system GroES/GroEL [2].

In this study, we report the construction of truncated variants and the search for an efficient manufacturing process for the production of *trans*-4-Hyp. To obtain a more soluble protein without use of chaperones, we predicted the secondary structure of *trans*-4-proline hydroxylase and removed some of the N-terminal or C-terminal amino acids. This is the first case in which the full-length gene shortening has been reported to improve activity of the enzyme. Additionally, we optimized the main factors influencing protein expression in *E. coli*, i.e. concentration of the inducer, the cell density before the inducer was added, the induction temperature, and the induction time, all of which enhanced the enzymatic activity. After optimization, the conversion efficiency was more than 97% within 48 h.

2. Materials and methods

2.1. Plasmid construction

The S-tag and thrombin recognition sites of the pET32a vector (Novagen, San Diego, CA) were replaced with a sequence encoding a rhinovirus 3C protease (3C) cleavable segment; we named this vector pET-32M-3C. The Trx fusion tag was deleted in pET-32M-3C, and we named this vector pET-M-3C.

Due to the codon usage and high G+C content of the proline 4-hydroxylase gene (GenBank No. D78338.1) from *Dactylosporangium sp.* strain RH1, codon usage at the 5′ end was optimized according to Shibasaki et al. (2000) [12]. The entire coding region of the gene was optimized and synthesized by Sangon Biotech, Shanghai (Supplemental Fig. S1 in [13]). The gene was then amplified through Polymerase Chain Reaction (PCR) with different primers for each of the following vectors: pET-M-3C, pET-32M, pUC18, and pTTQ18. The DNA fragments were digested and ligated into the corresponding vectors.

For the 1-257 aa (amino acid) fragment of proline 4-hydroxylase, the DNA oligomers S1 [5′-GTGAATTCATGCTGACCCCGACCGAACTG- 3′] and S2 [5′-CTCTCGAGCTAGGTGGCGTCACGAG CAGC-3′] were synthesized. The fragment was amplified with the full-length optimized gene serving as a template with an *EcoRI* restriction site incorporated at the 5′-end and an *XhoI* restriction site included at the 3′-end. The gene was digested with *EcoRI* and *XhoI* and ligated into the vector pET-M-3C to obtain fragment HYD_{1-257}.

The other truncated fragments of HYD, including 1-240, 5-240, 5-257, 5-272, 15-240, 15-257, 15-272, 25-240, 25-257, and 25-272, were ligated individually into pET-M-3C following the same procedure as for HYD_{1-257} except for the use of a different primer.

2.2. Protein solubility test

The production of HYD and HYD_{1-257} in *E. coli* BL21 CodonPlus (DE3) cells was performed in 5 mL of LB medium containing ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL) at 37°C, with shaking at 140 rpm. Isopropyl-β-D-thiogalactoside (IPTG) was added to the culture until the OD_{600} reached 0.6; the final IPTG concentration was 0.1 mM. Twenty μL of the bacterial suspension was collected before the inducer IPTG was added; we termed these samples *HYD before induction* and HYD_{1-257} *before induction*. After the cells were further cultured for 4 h at 20°C, 20 μL of the bacterial suspension was collected; we termed these samples *HYD after induction* and HYD_{1-257} *after induction*. The remaining bacterial suspension was collected by centrifugation (8000 × g, 15 min) and resuspended in 1 mL of lysis buffer (50 mM Tris-Cl, pH 7.0, 1 mM DTT, 1 mM EDTA, and 1 mg/mL lysozyme). The cells were lysed on ice for 30 min and centrifuged (12000 rpm, 15 min). Twenty μL of the supernatant was obtained, and we named these samples *HYD supernatant* and HYD_{1-257} *supernatant*. After the supernatant was discarded, the pellets were resuspended in 1 mL of lysis buffer. Pellets totaling 20 μL were obtained (*HYD pellet* and HYD_{1-257} *pellet*). The aforementioned samples were added to 5 μL of 5× loading buffer and boiled in a water bath at 100°C for 10 min. Finally, 10 μL of each sample was loaded onto a 12% SDS-PAGE gel for electrophoresis.

2.3. Primary protein expression and enzyme assay

The main cultures were grown in 500-mL flasks containing 200 mL of LB medium supplemented with ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL) on a rotary shaker at 140 rpm. *E. coli* BL21 CodonPlus (DE3) cells harboring the expression plasmid were grown in LB medium at 37°C until the OD_{600} reached 0.6. Subsequently, IPTG was added to the culture to reach a final concentration of 0.1 mM at 20°C for 4 h. After centrifugation at 5000 rpm for 15 min, 1 g of wet *E. coli* cells (OD_{600}=35) was resuspended in 10 mL of reaction buffer (80 mM MES pH 6.5, 200 mM L-proline, 200 mM α-oxoglutarate, 6 mM ferrous sulfate, 6 mM L-ascorbate, and 1% Nonidet P-40). The buffer composition was used, with some modifications, referring to what Shibasaki et al. (2000) reported. The reactions occurred in a 50-mL flask with shaking at 28°C. During the reaction, 400-μL samples

were centrifuged and the supernatant was prepared for High Performance Liquid Chromatography (HPLC). The conversion efficiency was observed from 24 to 60 h at 12 h intervals. Each experiment was repeated 3 times.

2.4. Effects of different concentrations of IPTG in E. coli on the enzymatic activity

The *E. coli* cells were cultured as described in Materials and Methods, Section 2.3; however, the cells were induced with 0, 0.05, 0.1, 0.2 and 0.3 mM IPTG at 20°C for 4 h, respectively.

2.5. Effects of cell density on the enzymatic activity prior to IPTG induction in E. coli

The *E. coli* cells were cultured, and the enzymatic activity was assayed as described in Materials and Methods section 2.3; however, the cells were grown in LB medium at 37°C until the OD_{600} reached approximately 0.6, 0.9, and 1.0. The cells were then induced with 0.2 mM IPTG at 20°C for 4 h.

2.6. Effects of the induction temperature on the enzymatic activity in E. coli

To identify the optimal induction temperature for maximal enzymatic activity in *E. coli*, the cells were cultured and the enzyme was assayed as described in Materials and Methods section 2.3; however, the cells harboring the expression plasmid were grown in LB medium at 37°C until the OD_{600} reached 0.9 and were then induced with 0.2 mM IPTG. The cells were further cultured at 20°C, 24°C, 28°C, and 37°C for 4 h.

2.7. Effects of the induction time on the enzymatic activity in E. coli

The cells were cultured and the enzyme was assayed as described in Materials and Methods section 2.3 with the exception that the cells were grown in LB medium at 37°C until the OD_{600} reached approximately 0.9. The cells were then induced with 0.2 mM IPTG at 28°C for 2, 4, 5, 6, 7, 9, 11, 13, and 15 h.

2.8. Derivatization and HPLC assay

The HPLC assay was conducted based on the following method [2,12,14] with some modifications. *Trans*-4-Hyp and L-proline were analyzed by HPLC using a Wondasil C18 column (4.6×250 mm, 5 µm) after precolumn derivatization. A total of 100 µL of sample solution was added to 2 mL of 120 mM sodium tetraborate buffer; 400 µL of 9-fluorenyl-methoxycarbonyl chloride (FMOC-Cl, 0.07 mM in acetonitrile) was then added, and the mixture was vortexed for 2 min. The total mixture was added to 5 mL of distilled water and filtered with a 0.22 µm filter. Samples of 10 µL were loaded onto the column, eluted at a flow rate of

1.0 mL/min, and analyzed by HPLC with the following conditions:

Mobile phase A: 0.1% trifluoroacetic acid (TFA)
　　　　　　　　B: acetonitrile.

Gradient　　　80% mobile phase A and 20% mobile phase B, 0-5 min;

　　　　　　　70% mobile phase A and 30% mobile phase B, 5-6 min;

　　　　　　　35% mobile phase A and 65% mobile phase B, 6-20 min;

　　　　　　　Maintain 35% mobile phase A, 20-28 min;

　　　　　　　80% mobile phase A and 20% mobile phase B during re-equilibration.

The derivatized *trans*-4-Hyp and proline were detected at an excitation wavelength of 243 nm and an emission wavelength of 263 nm. L-proline (Baokang Biotechnology Co., Ltd., Shijiazhuang) and *trans*-4-Hyp (Baokang Biotechnology Co., Ltd., Shijiazhuang) were purchased as standard substances. The product remaining after enzyme catalysis was defined based on the standard substances L-proline and *trans*-4-Hyp using HPLC. The retention time of L-proline was 23.34 min, and that of *trans*-4-Hyp was 18.41 min.

3. Results

3.1. Cloning and expression of recombinant proline hydroxylases and truncation activity tests

Codon optimization for *E. coli* was applied (GenBank No. KM088069) due to the codon usage and the G+C-rich content of the proline 4-hydroxylase gene from *Dactylosporangium sp.* strain RH1 (GenBank No. D78338.1). The full-length gene was ligated into four expression vectors with different promoters and fusion tags, and the recombinant plasmid was transformed into *E. coli* BL21-CodonPlus (DE3) for protein expression. The enzymatic activity was determined by HPLC after FMOC derivatization, and the conversion represented here is the concentration of Hyp after the transformation/concentration of (Hyp+L-Pro) after transformation at the defined time. Three repetitions of each test were conducted. The retention time (Figure 1(a), peak 1) of the product *trans*-4-hydroxyproline was the same as that of the standard substance (Figure 1(b), peak 1). We identified the product (Figure 1(a), peak 2) as proline and the substrate as the remaining substance that was not completely catalyzed by the enzyme; its retention time was the same as that of the standard substance (Figure 1(b), peak 2).

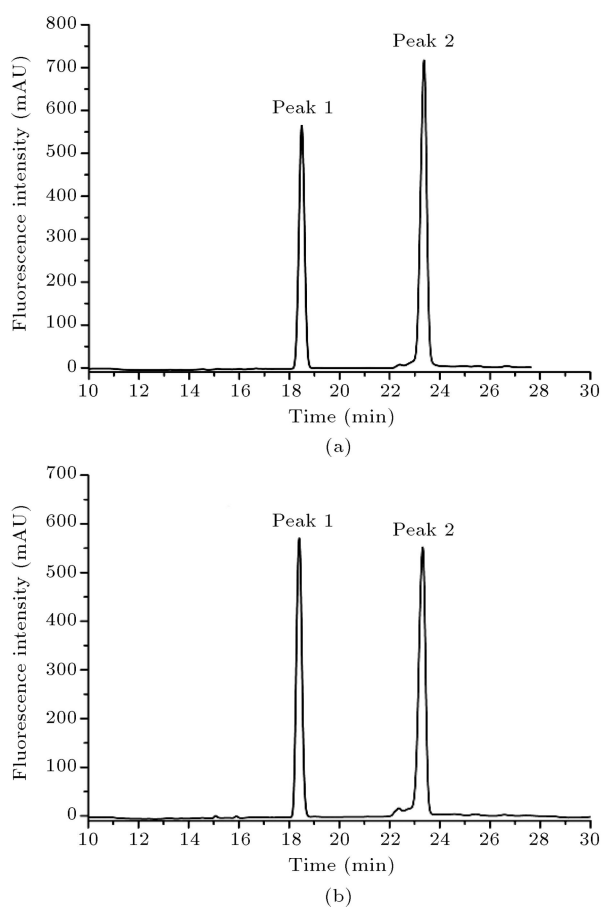In the enzymatic activity assay, little or no

Figure 1. Detection of *trans*-4-hydroxy-L-proline by HPLC. The HPLC conditions are described in the Materials and Methods section: (a) Analyzing a standard mixture containing *trans*-4-hydroxy-L-proline (peak 1) and L-proline (peak 2); and (b) analyzing the supernatant of the whole-cell reaction mixture by HPLC. Conversion is (the area of peak 1)/(the area of peak 1 + peak 2).

Table 1. Characterization of the four expression vectors and the *trans*-4-Hyp conversion rate.

Vector	Fusion tag	Promoter	Conversion (%) in 60 h
pET-M-3C	His$_6$	T7	76.60
pET-32M-3C	Trx -His$_6$	T7	61.47
pUC18	None	lac	0.14
pTTQ18	None	Tac	31.31

activity was detected except for the in-house-modified plasmids pET-M-3C (conversion: 76.60% in 60 h) and pET-32M-3C (conversion: 61.47% in 60 h) (Table 1). As pET-M-3C had no Trx fusion tag, we chose it as an expression vector for protein expression. We predicted the secondary structure of proline 4-hydroxylase (http://www.bioinf.manchester.ac.uk/dbbrowser/bioactivity/NPS2.html) and found that some amino acids of the N-terminal or C-terminal ends of proline

Table 2. Hydroxyproline conversion rates among the 12 constructs.

Fragment	Convert ratio (%) in 60 h
1-272	76.60±0.02
1-240	0.26±0.00
1-257	88.97±0.03
5-240	0.01±0.00
5-257	0.18±0.00
5-272	6.83±0.00
15-272	0.06±0.00
15-257	0.48±0.00
15-240	0.77±0.00
25-257	0.59±0.00
25-272	0.26±0.00
25-240	0.41±0.00

4-hydroxylase did not participate in the formation of the α-helix or formed a small α-helix (Supplemental Fig. S2 in [13]). The amino acids of the N-terminus, C-terminus, or both were deleted by PCR, and the fragments were constructed into pET-M-3C. The enzyme fragments were tested (Table 2) to determine whether they helped to improve the enzymatic activity. After the enzymes were assayed as described in Section 2.2, fragment 1-257 aa was found to have the highest conversion efficiency.

3.2. Comparing the solubility of HYD and HYD$_{1-257}$

Because an almost insoluble protein was obtained without the chaperone system in *E. coli* [2], and because the conversion efficiency of HYD$_{1-257}$ was higher than that of HYD, we tested whether the protein expression level and solubility were improved by truncation. As shown in Figure 2(a), there was not a large difference between the expression levels of HYD and HYD$_{1-257}$; however, we found that there was more truncated protein in the supernatant than in the assay with the full-length protein (Figure 2(b)). The enzymatic activity improved after the truncation, potentially, because there was a greater amount of soluble enzyme available to catalyze the substrate conversion to the product. Therefore, HYD$_{1-257}$ was used to optimize the enzyme expression conditions and improve the conversion efficiency.

3.3. Effects of the main influencing factors in the cultivation of the engineered strain on enzymatic activity

Because the enzyme was expressed in recombinant E. coli, the effects of the main influencing factors in the cultivation of the engineering strain on enzymatic activity were tested. We optimized the concentration of the inducer, cell density before the inducer was added, induction temperature, and induction time.
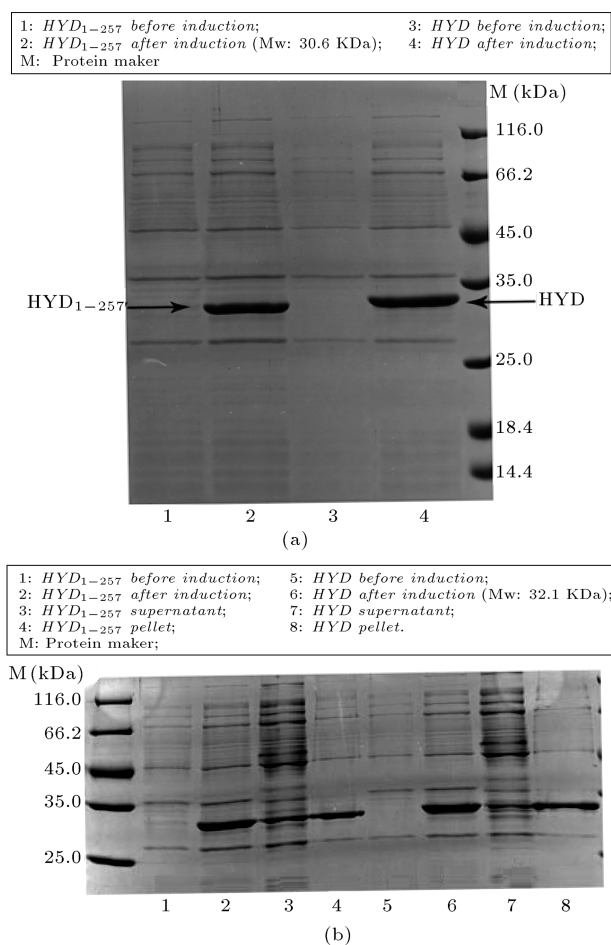
1: HYD_{1-257} *before induction*;   3: *HYD before induction*;
2: HYD_{1-257} *after induction* (Mw: 30.6 KDa);   4: *HYD after induction*;
M: Protein maker

1: HYD_{1-257} *before induction*;   5: *HYD before induction*;
2: HYD_{1-257} *after induction*;   6: *HYD after induction* (Mw: 32.1 KDa);
3: HYD_{1-257} *supernatant*;   7: *HYD supernatant*;
4: HYD_{1-257} *pellet*;   8: *HYD pellet*.
M: Protein maker;

Figure 2. HYD and HYD_{1-257} expressions (a), and the protein solubility comparison (b).
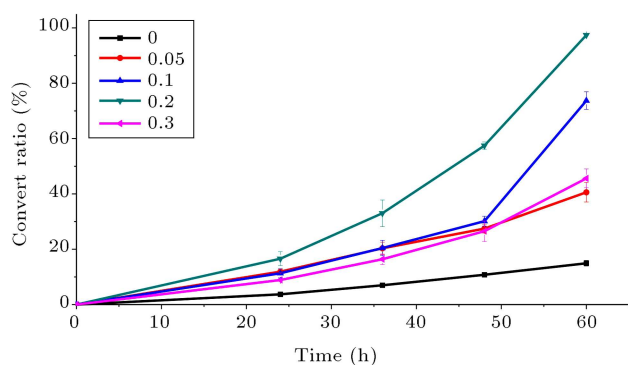

Figure 3. *trans*-4-Hyp conversion efficiency at different concentrations of IPTG.

The expression of HYD genetically engineered bacteria was induced by IPTG. The initial concentration of IPTG may affect the enzymatic activity and the final production of *trans*-4-Hyp; therefore, the effect of the initial concentration of IPTG was tested. Figure 3 shows the *trans*-4-Hyp production under various concentrations of IPTG. The highest *trans*-4-Hyp production at an initial IPTG concentration of 0.2 mM was 57.44% at 48 h and 97.4% at 60 h. These
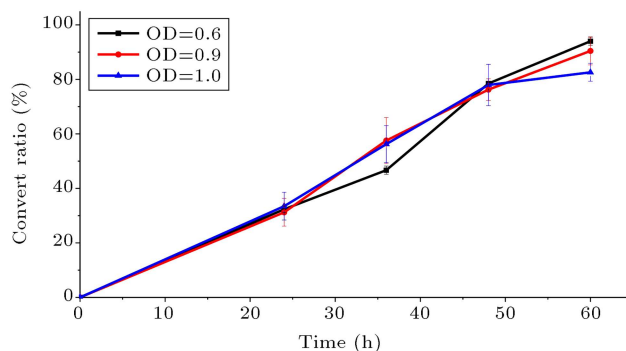

Figure 4. *trans*-4-Hyp conversion efficiency at different cell densities before IPTG was added.

results demonstrate that an IPTG concentration of 0.2 mM is optimal for the production of *trans*-4-Hyp.

The cell density, added before the inducer, was also evaluated to determine whether this parameter affected the enzymatic activity. The enzyme was induced at different OD_{600} values and was analyzed by HPLC (Figure 4). The enzyme conversion efficiency was >90% when the OD_{600} reached 0.6 or 0.9 within 60 h. The total number of cells was high when the OD600 reached 0.9, and therefore, we chose a cell density corresponding to an OD_{600} of 0.9 to evaluate the enzyme expression.

Because the characteristics of each target gene product are unique, the expression conditions for each protein can vary. For example, growth at 37°C causes some proteins to accumulate as inclusion bodies, whereas incubation at 30°C can lead to the production of soluble, active proteins. Different induction temperatures may affect the correct folding of recombinant proteins [15-18]. Therefore, we sought to determine the most efficient temperature for this enzyme. Figure 5 shows the HYD_{1-257} activities that were detected at induction temperatures ranging from 20 to 37°C. The conversion efficiency was tested by HPLC from 24 to 60 h at 12 h intervals. It was evident that the conversion efficiency differed at different temperatures: the conversion ratio was 98.57% at 28°C and only 67.01% at 37°C after 60 h. As 28°C, i.e. approximately
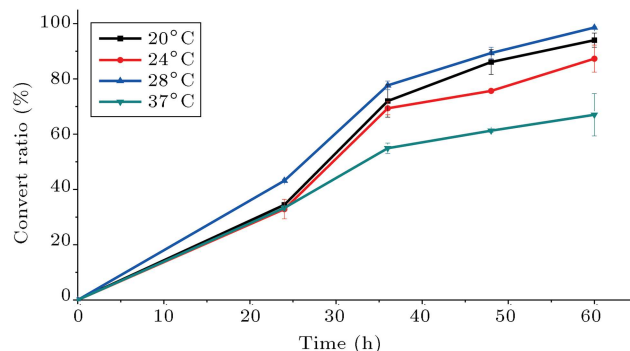

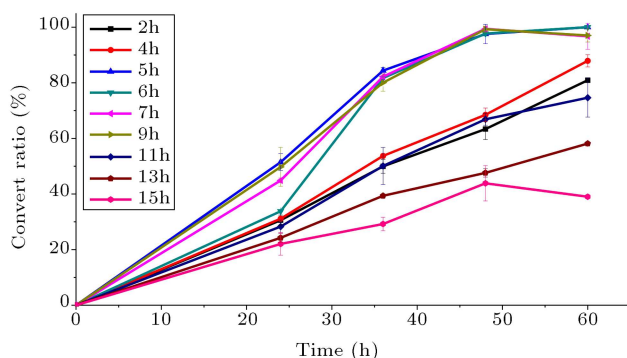**Figure 5.** *trans*-4-Hyp production at different induction temperatures.

Figure 6. *trans*-4-Hyp conversion efficiency at different induction times.

room temperature, showed the highest conversion ratio, we chose 28°C to induce protein expression in our experiment.

The effect of different induction times on *trans*-4-Hyp production in *E. coli* is shown in Figure 6. The conversion efficiency of *trans*-4-Hyp was > 97% within 48 h for an induction time of 5-9 h. The conversion efficiency of *trans*-4-Hyp was 74.59%, 58.11%, and 39.02% with induction times of 11, 13, and 15 h, respectively, after 60 h. The conversion efficiency was reduced with an induction time longer than 9 h. These results indicate that an induction time of approximately 5-9 h is favorable for *trans*-4-Hyp production and productivity. The cell density was higher with an induction time of 9 h compared with 5 h, and thus, we chose to induce the engineered strain for 9 h for optimal protein expression.

4. Discussion

To obtain a larger quantity of active enzyme and determine the most efficient process for manufacturing *trans*-4-Hyp, a suitable expression vector was necessary, and the length of the enzyme was evaluated [12,19-21]. The cultivation conditions for the engineered strain were also optimized [22,23]. In our work, we tested the enzymatic activities in four different vectors with different fusion tags and promoters, and we found that the pET system was most suitable for enzyme expression. Additionally, in an attempt to improve the activity of the enzyme, 11 truncated constructs were created and ligated into the in-house-modified pET-M-3C vectors. The truncated 1-257 aa was found to have the highest conversion efficiency. We found that there was a greater amount of soluble enzyme than that of the full-length enzyme in the supernatant when using HYD_{1-257}. The C-terminal 258-272 aa was predicted to form random coils because there were three prolines and three glycines among the 15 C-terminal amino acids (PLPAGFALAQPV), and both amino acids were helix stop signals [24]. We hypothesize that the increased solubility is the main factor leading to the

increased enzymatic activity. As for the other 10 truncated constructs, we found that their expression level was reduced to varying degrees. There is the possibility of one common reason for the decline in their activity. Further investigation is needed to determine whether their solubility was changed. This is the first report of the successful expression of truncated active HYD_{1-257} in a bacterial expression system that has more activity than the full-length HYD.

HYD_{1-257} was used to optimize the enzyme expression conditions to improve the conversion efficiency. The most suitable conditions for protein expression involved the addition of 0.2 mM IPTG to the engineered strain when the OD600 reached 0.9 with induction at 28°C for 5-9 h. The conversion efficiency was more than 97% within 48 h.

During our research, we found that approximately 14% of the proline was lost due to metabolization by *E. coli*, slightly more than what Shibasaki et al. (2000) reported (13%). This loss can be reduced by deletion of the proline dehydrogenase gene *putA* in *E. coli* [2,12]. Our yield was 109 mg *trans*-4-Hyp per 100 mL cell culture, and was more than 3 times higher than what Klein et al. (2011) [2] reported. Though our yield did not reach that reported by Shibasaki et al. (2000) [12], we provided a more active fragment than the full-length gene. It is possible that Shibashi et al. (2000) could shorten their fermentation time (100 h) by shortening the full-length gene. We will construct a *putA* mutant *E. coli* to improve our yield in further research.

Acknowledgment

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