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Abstract

Objective: To express heterologous D-hydantoinase gene (hyd) and D-carbamoylase gene (adc) in Bacillus subtilis, constructing recombinant cells as catalysts for the production of D-p-hydroxyphenylglycine (D-HPG). Methods: hyd expression plasmids were constructed to investigate the effects of divalent metal ions in the medium on D-hydantoinase activity. The acoR gene was overexpressed to examine the relationship between intracellular AcoR protein levels and PacoA-hyd gene copy number. Promoters for adc gene expression were screened, hyd and adc gene co-expression plasmids were constructed, and the catalytic characteristics of the dual-enzyme active strain were evaluated. Results: The hydantoinase expression plasmids pHPS and pUBS were successfully constructed; supplementation of the medium with 0.8 mmol/L $MnCl2 \cdot 4H2O$ increased the D-hydantoinase activity of strain 168N/pUBS to 956 U/gDCW. Integrated expression of the Pcdd-acoR gene elevated the D-hydantoinase activity of strain LSL02/pUBS to 1470 U/gDCW. The single-copy PAE-adc gene exhibited the relatively highest expression level. The dual-enzyme co-expression plasmid pUBSC was successfully constructed; strain LSL02/pUBSC exhibited an optimal catalytic temperature of 40-45°C, and catalytic activity could be maintained for 12 h. When the initial substrate concentration was 20 g/L, D-HPG production reached 14.32 g/L after 12 h of reaction, with a conversion rate of 95% and a yield exceeding 80%. Conclusion: The construction of recombinant Bacillus subtilis with dual D-hydantoinase and D-carbamoylase activities as a whole-cell catalyst for the hydantoinase-based production of D-HPG is technically feasible and advantageous.

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Full Text

Construction of Recombinant *Bacillus subtilis* as a Catalyst for Preparing D-p-Hydroxyphenylglycine

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Abstract

Objective: To express heterologous D-hydantoinase gene (*hyd*) and D-carbamoylase gene (*adc*) in *Bacillus subtilis*, constructing recombinant cells as catalysts for the production of D-p-hydroxyphenylglycine (D-HPG). **Methods:** D-hydantoinase expression plasmids were constructed, and the effects of divalent metal ions in the culture medium on D-hydantoinase activity were investigated. The *acoR* gene was overexpressed to examine the relationship between intracellular AcoR protein levels and *PacoA-hyd* gene copy number. Promoters for *adc* gene expression were screened, a dual-enzyme co-expression plasmid was constructed, and the catalytic properties of the resulting strain were characterized. **Results:** The hydantoinase expression plasmids pHPS and pUBS were successfully constructed. When 0.8 mmol/L $MnCl_2 \cdot 4H_2O$ was added to the medium, the D-hydantoinase activity of strain 168N/pUBS reached 956 U/gDCW. Integration of the *Pcdd-acoR* gene increased the D-hydantoinase activity of strain LSL02/pUBS to 1470 U/gDCW. Single-copy *PAE-adc* gene expression showed the highest relative expression level. The dual-enzyme co-expression plasmid pUBSC was successfully constructed. Strain LSL02/pUBSC exhibited an optimal catalytic temperature of 40–45°C, with catalytic activity sustained for 12 h. When the initial substrate concentration was 20 g/L, the D-HPG concentration reached 14.32 g/L after 12 h reaction, with a conversion rate of 95% and a yield exceeding 80%. **Conclusion:** The construction of recombinant *Bacillus subtilis* possessing both D-hydantoinase and D-carbamoylase activities as a whole-cell catalyst for hydantoinase-based D-HPG production is technically feasible and advantageous.

Keywords: *Bacillus subtilis*; D-hydantoinase; D-carbamoylase; expression plasmid; D-p-hydroxyphenylglycine

D-p-hydroxyphenylglycine (D-HPG) serves as a pharmaceutical intermediate primarily used in the semi-synthesis of antibiotics such as amoxicillin, cefadroxil, and cefoperazone [1]. The hydantoinase process for D-HPG production uses D,L-p-hydroxyphenylhydantoin (D,L-HPH) as substrate, which is converted to optically pure D-HPG through the intermediate N-carbamoyl-D-p-hydroxyphenylglycine (D-CpHPG) under the catalysis of D-hydantoinase (D-hydantoinase, DHase) and D-carbamoylase (D-carbamoylase, DCase) (Fig-

ure 1 [Figure 1: see original paper]) [2-3]. Under alkaline reaction conditions, D,L-HPH can spontaneously racemize [4], enabling complete conversion of D,L-HPH without requiring a separate racemase.

D-hydantoinase (EC 3.5.2.2) belongs to the amidohydrolase family and is found in microorganisms such as *Brevibacillus*, *Bacillus*, *Agrobacterium*, and *Pseudomonas* [5]. Microbial D-hydantoinases are typically homodimers or homotetramers with subunit molecular weights of 40–60 kDa and approximately 450 amino acid residues [6-7]. The active center of most D-hydantoinases consists of His, Asp, Val, and carboxylated Lys residues, exhibiting catalytic activity toward D-5’ -substituted hydantoins. The two oxygen atoms of the carboxylated Lys coordinate with two Zn^{2+} ions, making D-hydantoinase a metal-dependent enzyme [8]. When D,L-HPH serves as substrate, the specific activity of D-hydantoinase typically ranges from 10 to 100 U/mg. The D-hydantoinase from *Bacillus stearothermophilus* SD-1 (*hyd* gene) demonstrates exceptional thermostability with a half-life of 30 min at 80°C, a Km value of 47.2 mM, and specific activity of approximately 23.0 U/mg [9], making it suitable for D-HPG production via the hydantoinase process and often the preferred choice.

D-carbamoylase (EC 3.5.1.77) has been identified in various microorganisms including *Agrobacterium*, *Arthrobacter*, *Blastobacter*, *Comamonas*, *Pseudomonas*, *Sinorhizobium*, *Flavobacterium*, and *Pasteurella* [10], and also belongs to the amidohydrolase family. D-carbamoylase exhibits stereoselectivity, specifically catalyzing the hydrolysis of the amide bond in N-carbamoyl-D-amino acids to produce corresponding D-amino acids. D-carbamoylases are predominantly homotetramers with subunit molecular weights around 35 kDa, containing over 300 amino acid residues, with active centers composed of Glu46, Lys126, and Cys171 residues. However, studied D-carbamoylases suffer from poor thermostability and susceptibility to oxidative inactivation, with specific activities typically ranging from 7 to 22 U/mg. The D-carbamoylase from *Agrobacterium* sp. KNK712 [11] (*adc* gene) is often selected for dual-enzyme catalytic production of D-HPG due to its well-characterized enzymatic properties.

The hydantoinase process for D-HPG production can be categorized into two main approaches: one using immobilized enzymes or immobilized cells as catalysts, and the other using free whole cells as catalysts. The latter offers advantages of simpler catalyst preparation and lower cost, showing better application prospects. Naturally occurring microorganisms with dual-enzyme activity cannot meet application requirements due to low enzyme activities. Mutant strains obtained through breeding and screening often exhibit growth defects or low biomass, also failing to satisfy industrial needs. Genetic engineering technology provides a viable alternative for constructing recombinant strains. To date, almost all recombinant strains constructed for expressing D-hydantoinase and D-carbamoylase have used *Escherichia coli* as the host [12-14]. Although recombinant *E. coli* whole-cell catalytic activity can meet application requirements, large-scale cultivation faces technical challenges such as dissolved oxygen limitations, inducer costs, and inclusion body formation [15-16], restricting its

industrial application. Recombinant *Bacillus subtilis* may represent a better alternative.

Previous studies have confirmed that heterologous D-hydantoinase and D-carbamoylase can be expressed in *Bacillus subtilis* [17]. This study further optimizes the expression strategies and conditions for both enzymes, refines the construction of dual-enzyme co-expressing recombinant *B. subtilis* strains, and characterizes the whole-cell catalytic properties of the resulting recombinants.

1.2 Culture Media and Strain Cultivation Methods

LB medium (g/L): peptone 10, yeast extract 5, NaCl 10, pH 7.5; supplemented when necessary with neomycin (15 mg/L), chloramphenicol (6 mg/L), or bleomycin (50 mg/L) (Beijing Solarbio Science & Technology Co., Ltd.). SPM medium (g/L): roasted soybean powder 20, yeast powder 5, $MgSO_4 \cdot 7H_2O$ 0.3, K_2HPO_4 2, NaCl 5, $MnCl_2 \cdot 4H_2O$ 0.16, pH 7.2. LB medium was used for strain activation, seed culture, and general cultivation, while LB or SPM medium was employed for D-hydantoinase and D-carbamoylase expression and whole-cell catalytic activity measurement. Cultures were grown in 250 mL flasks containing 30 mL medium, inoculated with 2% of a 12 h seed culture, and incubated at 37°C with shaking at 220 rpm. Acetoin (3 g/L) was added at 6 h to induce D-hydantoinase gene expression. Biomass was determined by microscopic total cell counting, with 1.42$\times 10^{12}$ cells equivalent to 1.0 g DCW (dry cell weight).

1.3 Genetic Manipulation Methods

Primer synthesis and DNA sequencing were performed by Beijing Aoke Dingsheng Biotechnology Co., Ltd. All other DNA fragments were obtained by PCR amplification from *Bacillus subtilis* chromosome using Phanta Max Super-Fidelity DNA Polymerase (Nanjing Vazyme Biotech Co., Ltd.) and Hifi DNA Polymerase (Beijing TransGen Biotech Co., Ltd.). dNTPs were purchased from Thermo Scientific. PCR-based fusion of DNA fragments followed the method described in reference [18]. Restriction endonucleases and T4 DNA ligase were obtained from Thermo Scientific and used according to manufacturer instructions. The Multifunctional DNA Purification Kit was purchased from Beijing Biomed Biotechnology Co., Ltd. Competent cell preparation and transformation were performed using the Spizizen method [19]. Gene integration into the *B. subtilis* chromosome employed a marker-free modification method [20]. Integration of *Pcdd-adc* at the *pel* locus serves as an example: the upstream homologous arm (U, 1,261 bp) and the promoter plus leader region of the *cdd* gene (*Pcdd*, 232 bp) were amplified from *B. subtilis* 168 chromosome using primers *pelU1/pelU2* and *AP1s/AP2s*, respectively; the *adc* gene (N, 1,128 bp) was amplified from plasmid pHG-cdh using primers *peladcA/peladcB*; and the internal homologous fragment, forward selection cassette, and downstream homologous arm (DCRG, 3,320 bp) were amplified from *B. subtilis* BNAY6m chromosome using primers *pelD1/pelG2*. These four fragments were fused via fusion PCR

to generate the UPNDCRG fragment (5,941 bp), which was used to transform competent *B. subtilis* 168N cells. Chloramphenycin-resistant strains were selected on appropriate plates and verified by PCR amplification and resistance testing. Chloramphenicol-resistant strains were then transferred to LB medium and cultured at 37°C with shaking at 220 rpm for 4 h, followed by selection on neomycin plates. Transformants were verified by PCR amplification and DNA sequencing to obtain the recombinant strain LN01 with *Pcdd-adc* integrated at the *pel* locus. All other gene or DNA fragment integrations into the *Bacillus subtilis* chromosome employed the same method. Table 2 lists the primers used in this study.

1.4 qRT-PCR Analysis Method

Strains were cultured in LB medium, and cells were harvested at 16 h for total RNA extraction (Tiangen Biotech extraction kit). The first cDNA strand was synthesized (Roche) and RT-PCR amplification was performed (Roche Light Cycler 480) to determine Ct values. Using *B. subtilis ccpA* as the reference gene, relative mRNA levels were calculated according to the $2^{-}\Delta\Delta$Ct method [21].

1.5 Intracellular Protein Extraction and SDS-PAGE Analysis

Two milliliters of fermentation broth were centrifuged at 13,000 rpm for 2 min, the supernatant was removed, and the cell pellet was washed thoroughly. The cells were resuspended in 500 L sterile water, transferred to a disruption tube with 200 L glass beads, and disrupted using a homogenizer (FastPrep FP120). Disruption consisted of 30 cycles of 45 s disruption followed by 2 min on ice. The cell lysate was centrifuged at 6,000 rpm for 8 min at 4°C, and the supernatant was mixed with 4 volumes of cold absolute ethanol to precipitate proteins. After centrifugation at 13,000 rpm for 8 min, the supernatant was discarded and the pellet was dried at 37°C. The precipitate was dissolved in 30 L sterile water to obtain the intracellular protein extract. An equal volume of 2× loading buffer was added, mixed thoroughly, heated in a boiling water bath for 10 min, cooled, and centrifuged to collect the sample at the tube bottom. Twenty microliters were loaded for SDS-PAGE analysis.

1.6 D,L-HPH Conversion Reaction and Whole-Cell Catalytic Activity Determination

D,L-HPH and D-HPG concentrations were determined by high-performance liquid chromatography (Agilent 1260 Infinity Quaternary LC) using an Agilent ZORBAX SB-C18-5 m (4.6 mm × 150 mm) column. The mobile phase consisted of 50 mM sodium acetate-acetic acid buffer (pH 4.2) and methanol at a 90:10 volume ratio, with a flow rate of 0.5 mL/min, injection volume of 10 L, column temperature of 30°C, and UV detection at 254 nm.

D-hydantoinase activity assay: 0.2 mL cell culture was centrifuged at 13,000

rpm for 3 min to collect cells, which were washed twice with 50 mmol/L Tris • HCl buffer (pH 8.0) and resuspended in 2 mL of 0.3% D,L-HPH (dissolved in Tris • HCl buffer). The reaction was performed at 40°C with shaking for 30 min, then terminated by adding 10 L of 6 mol/L HCl. The supernatant was analyzed by HPLC to quantify D,L-HPH consumption. Enzyme activity was calculated using the formula: U/gDCW = $1.42 \times c \div (192.17 \times 30 \times n) \times 10^4$, where c represents D,L-HPH consumption (mg/L) and n represents biomass per mL fermentation broth ($×10^{9}$ cells). One unit (U) of D-hydantoinase activity was defined as the amount of enzyme required to hydrolyze 1 mmol/L of D-HPH to D-CpHPG per minute per gram of dry cells.

D-carbamoylase activity assay: Two milliliters of cell pellet were resuspended in an equal volume of 15 mmol/L D-CpHPG reaction solution and incubated at 40°C with shaking for 60 min. The reaction was terminated by adding 10 L of 6 mol/L HCl. The supernatant was analyzed by HPLC to quantify D-HPG production. Enzyme activity was calculated using the formula: U/gDCW = $1.42 \times c \div (167.16 \times 60 \times n) \times 10^3$, where c represents D-HPG production (mg/L) and n represents biomass per mL fermentation broth ($×10^{9}$ cells). One unit (U) of D-carbamoylase activity was defined as the amount of enzyme required to hydrolyze D-CpHPG to produce 1 mmol/L of D-HPG per minute per gram of dry cells.

Dual-enzyme catalytic activity assay for whole-cell catalysts followed the D-hydantoinase activity determination method. One unit of dual-enzyme catalytic activity was defined as the amount of enzyme required to hydrolyze D-HPH to produce 1 mmol/L of D-HPG per minute per gram of dry cells.

2.1 Expression of D-Hydantoinase Gene in *Bacillus subtilis*

The D-hydantoinase gene *hyd* was amplified from plasmid pHG-sd1 to replace the coding sequence of the *acoA* gene in the *B. subtilis* 168N chromosome, creating the integrated *PacoA-hyd* gene and generating recombinant strain LS10. The *PacoA-hyd* gene was then amplified from the LS10 chromosome with *Hind*III and *Bam*HI restriction sites introduced at both ends and ligated with similarly digested plasmid pHP13 to form the low-copy D-hydantoinase expression plasmid pHPS (Figure 2a [Figure 2: see original paper]). Plasmid pHPS was transformed into *B. subtilis* 168N to obtain strain 168N/pHPS.

The replication region (*rep*), kanamycin resistance gene (*kmr*), and bleomycin resistance gene (*blm*) [22] were amplified from plasmid pUB110 and fused with the *PacoA-hyd* gene via PCR, with *Pst*I sites introduced at both ends. The fused sequence was digested and ligated to form the multi-copy D-hydantoinase expression plasmid pUBS (Figure 2b [Figure 2: see original paper]). Plasmid pUBS was transformed into strain 168N, and bleomycin-resistant transformants were selected to obtain strain 168N/pUBS.

Strains LS10, 168N/pHPS, and 168N/pUBS were cultured in LB medium, and 24 h cultures were harvested as whole-cell catalysts for D,L-HPH conversion re-

actions. After 60 min reaction, addition of 10% p-dimethylaminobenzaldehyde (PDAB) reagent produced a yellow color, indicating D-CpHPG formation. HPLC chromatograms showed (Figure 3a [Figure 3: see original paper]) that before reaction, only the D,L-HPH peak at retention time 7.60 min was present (blue line), while after 60 min, the D,L-HPH peak area decreased and a D-CpHPG peak appeared at 3.45 min (red line). Intracellular proteins from strains LS10, 168N/pHPS, and 168N/pUBS were extracted and analyzed by SDS-PAGE. Results showed (Figure 3b [Figure 3: see original paper]) prominent bands at approximately 54 kDa in strains 168N/pHPS and 168N/pUBS, corresponding to the D-hydantoinase monomer, with band intensity increasing with *PacoA-hyd* gene copy number. These results demonstrate that the *PacoA-hyd* gene can be expressed whether integrated into the chromosome or located on expression plasmids, conferring D-hydantoinase activity to host cells.

2.2 Effects of Divalent Metal Ions on D-Hydantoinase Activity

D-hydantoinase belongs to the metal-dependent enzyme subfamily. Crystal structure analysis of hydSD-1 reveals that each subunit' s carboxylated Lys150 binds two Zn^{2+} ions, while earlier studies showed the enzyme maintains stable activity in Mn^{2+}-containing buffers, suggesting divalent metal ions significantly influence activity. Different concentrations of $FeCl_2 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, and $MnCl_2 \cdot 4H_2O$ were added to LB medium for cultivating strain 168N/pUBS, with EDTA as control. Cells were harvested at 24 h, and biomass measurements ranged from 1.6$\times10^{9}$ to 2.0$\times10^{9}$ cells/mL, showing no significant growth differences. D-hydantoinase activity measurements (Figure 4 [Figure 4: see original paper]) revealed lowest activity in the EDTA control group, with activity decreasing as EDTA concentration increased. $FeCl_2$ slightly inhibited activity, $MgSO_4$ showed no significant effect, while $ZnSO_4$ exhibited clear activation but became inhibitory above 0.2 mmol/L. $MnCl_2$ demonstrated strong activation across 0.2-1.0 mmol/L, with 0.8 mmol/L $MnCl_2 \cdot 4H_2O$ increasing strain 168N/pUBS D-hydantoinase activity over 10-fold to 956 U/gDCW.

2.3 Relationship Between *acoR* Gene Expression Level and D-Hydantoinase Activity

PacoA is a σ -dependent promoter whose transcription requires activation by AcoR protein and induction by acetoin [23]. With multi-copy *PacoA-hyd* genes present, endogenous AcoR protein levels may be insufficient for full activation of all *PacoA-hyd* genes [24]. In strains LSL10 and LSL (both with dual *sigL* gene copies), a wild-type *acoR* gene copy was integrated at the chromosomal *sacB* locus to construct strains LSL11 and LSL01; a *Pcdd-acoR* (*acoR*) gene copy was integrated to construct strains LSL12 and LSL02; and a *PAE-acoR* (*acoR*) gene copy was integrated to construct strains LSL13 and LSL03 [25],

enabling varying degrees of *acoR* overexpression. qRT-PCR analysis (Table 3) showed that intracellular *acoR* mRNA levels in strains LSL01, LSL02, and LSL03 increased 2.11-, 63.56-, and 302.33-fold compared to control strain LSL, confirming differential *acoR* overexpression in these strains.

Strains LSL10–13 and LSL–03/pHPS and LSL–03/pUBS series were cultured in LB medium supplemented with 0.8 mmol/L $MnCl_2 \cdot 4H_2O$, and D-hydantoinase activity was measured in 24 h cells. Results (Figure 5 [Figure 5: see original paper]) showed that in strains LSL10–13 with single-copy *PacoA-hyd*, increasing *acoR* expression up to 60-fold had no effect on whole-cell D-hydantoinase activity, while a 300-fold increase dramatically reduced activity. In strains LSL–03/pHPS with low-copy *PacoA-hyd*, a 2-fold increase in *acoR* expression yielded maximum D-hydantoinase activity of 1230 U/gDCW. In strains LSL–03/pUBS with approximately 50 copies of *PacoA-hyd*, a 60-fold increase in *acoR* expression produced maximum activity of 1470 U/gDCW. These results demonstrate that intracellular AcoR protein levels and *PacoA-hyd* gene copy number must maintain an appropriate ratio for full activation of *PacoA-hyd* gene expression.

2.4 Expression of D-Carbamoylase Gene in *Bacillus subtilis*

The D-carbamoylase gene *adc* from *Agrobacterium* sp. KNK712 was amplified from plasmid pHG-cdh and fused with promoters *Pcdd*, *PspoVG*, *PlytR* [26], and *PAE*, then integrated into the *pel* gene locus of *B. subtilis* 168N chromosome to construct recombinant strains LN01, LN02, LN03, and LN04. qRT-PCR analysis (Table 4) revealed relative intracellular *adc* mRNA levels as: LN04 > LN02 > LN01 > LN03, with *PAE-adc* expression in LN04 being 60.55-fold higher than *Pcdd-adc* expression in LN01.

Strains LN01, LN02, LN03, and LN04 were cultured in LB medium supplemented with 0.8 mmol/L $MnCl_2 \cdot 4H_2O$, and D-carbamoylase activity was measured at different growth phases. Results (Figure 6 [Figure 6: see original paper]) showed highest activity in LN04 (approximately 8.0 U/gDCW at 17 h), followed by LN02 (approximately 6.5 U/gDCW at 14 h), while LN01 and LN03 activities were below 0.4 and 0.8 U/gDCW, respectively. Cellular D-carbamoylase activity positively correlated with *PAE-adc* expression level, demonstrating that the *PAE* promoter enables the highest D-carbamoylase activity.

2.5 Dual-Enzyme Co-expression and Effects of Medium Components on Catalytic Activity

Using pUB110 as the vector, the *PacoA-hyd* and *PAE-adc* genes were fused, digested, ligated to the vector, and transformed into LSL02 competent cells. Bleomycin-resistant transformants were selected to obtain strain LSL02/pUBSC, with recombinant plasmid pUBSC serving as the dual-enzyme co-expression plasmid (Figure 7 [Figure 7: see original paper]).

To investigate effects of different medium components on whole-cell catalytic ac-

tivity, SPM medium served as the control, with partial organic nitrogen sources replaced or carbon sources added, and catalytic activity of LSL02/pUBSC as whole-cell catalyst was measured. Results (Figure 8 [Figure 8: see original paper]) showed highest catalytic activity (64.6 U/gDCW at 22 h) when using SPM medium containing the completely slow-release nitrogen source combination of 2% roasted soybean powder and 0.5% yeast powder. Replacing yeast powder with 0.5% yeast extract yielded maximum activity of 44.0 U/gDCW at 24 h, while replacing roasted soybean powder with 2% peptone gave maximum activity of 22.0 U/gDCW at 20 h. Clearly, slow-release organic nitrogen sources favor higher catalytic activity, while fast-release sources reduce cellular catalytic activity. Both glucose (a PTS sugar) and glycerol (a non-PTS sugar) severely impaired catalytic activity.

2.6 Catalytic Characteristics of Strain LSL02/pUBSC

Strain LSL02/pUBSC was cultured in SPM medium, and 22 h cells were harvested as catalysts and resuspended in an equal volume of reaction solution with 3.0 g/L initial D,L-HPH concentration. Reactions were performed at 35°C, 40°C, 45°C, and 50°C. Results (Figure 9 [Figure 9: see original paper]) showed reduced reaction rates below 40°C, relatively high and equivalent rates between 40–45°C, and dramatically decreased rates at 50°C. Therefore, the optimal temperature for LSL02/pUBSC as whole-cell catalyst is 40–45°C.

HPLC analysis of the 40°C reaction process (Figure 10 [Figure 10: see original paper]) revealed D-CpHPG accumulation, indicating D-hydantoinase catalytic rate exceeded that of D-carbamoylase. After 6 h, substrate was 100% converted without intermediate accumulation, D-HPG concentration reached 2.34 g/L, and actual yield approached 90%.

With initial D,L-HPH concentrations increased to 10, 15, and 20 g/L, reactions were performed under standard conditions at a 2:1 cell-to-substrate ratio (8 mL cells concentrated in 4 mL reaction volume), with sampling every 2 h to monitor D-HPG concentration changes and substrate/intermediate accumulation. Results (Figure 11 [Figure 11: see original paper]) showed D-HPG concentrations of 7.65, 11.05, and 14.32 g/L after 12 h, with yields of 88.0%, 84.7%, and 82.4%, respectively. Process chromatograms revealed that at this catalyst loading, 100% conversion could be achieved at substrate concentrations below 15 g/L, though minor intermediate accumulation occurred at 15 g/L. At 20 g/L substrate, approximately 95% conversion was achieved with residual intermediate, and component concentrations remained unchanged with extended reaction time, indicating catalyst activity exhaustion after 12 h use.

Discussion

In *Bacillus subtilis*, heterologous D-hydantoinase from *Bacillus stearothermophilus* SD-1 was successfully expressed using the acetoin-inducible promoter *PacoA*, and both low-copy expression plasmid pHPS and high-copy expression

plasmid pUBS were constructed, demonstrating that *PacoA* is a reliable and convenient inducible expression element. Crystal structure studies of D-hydantoinase reveal two coordinated Zn^{2+} ions in the active center, while the enzyme maintains stable activity in Mn^{2+}-containing buffers. This study confirmed that Mn^{2+} addition to the medium activates D-hydantoinase more strongly than Zn^{2+}. Mn^{2+} occupies larger space in the active center than Zn^{2+}, potentially facilitating binding of D-5' -substituted substrates with larger groups such as D-HPH to the active site.

RNA polymerase and σ -recognized promoters form closed transcription complexes. AcoR protein binds to upstream regions of *PacoA* forming highly ordered oligomers, and ATP hydrolysis provides energy for conformational changes that transition the complex to an activated state capable of interacting with σ to activate transcription [27-28]. The intracellular AcoR protein level from single-copy *acoR* expression may only be sufficient to fully activate low-copy *PacoA* promoters. When approximately 50 copies of plasmid pUBS exist intracellularly, *acoR* gene overexpression is required to increase intracellular AcoR protein levels for full activation of all *PacoA* promoters and achieve high D-hydantoinase activity. Conversely, when *acoR* is overexpressed but *PacoA* promoter copy number is low, excess intracellular AcoR protein may alter binding to upstream *PacoA* elements or affect conformational transitions, hindering RNA polymerase binding to *PacoA* promoters and reducing D-hydantoinase activity. Therefore, an appropriate proportional relationship exists between intracellular AcoR protein levels and *PacoA* promoter copy number. Previous studies have also suggested this proportional relationship: Silbersack et al. [23] expressed the *amyE* gene using this system, integrating *acoR* expression on the chromosome and multicopy plasmid, which increased amylase activity by 2- and 3-fold, respectively. In contrast, Sophia Zobel' s research [29] showed that adding an extra copy of *acoR* and *sigL* nearly eliminated enniatin production.

PAE is a constitutive strong promoter, and transcriptional analysis confirmed high-level transcription of *PAE-adc*. Additionally, the specific activities of D-hydantoinase and D-carbamoylase are not substantially different. However, in LSL10 strain with single-copy inducible *PacoA-hyd*, whole-cell D-hydantoinase activity reached 500 U/gDCW, while single-copy *PAE-adc* constitutive expression in LN4 strain yielded only 8.0 U/gDCW D-carbamoylase activity—a two-order-of-magnitude difference. Although slow-release nitrogen sources like roasted soybean powder and yeast powder in the medium improved D-carbamoylase activity, the actual enhancement was limited. D-carbamoylase has poor thermostability and is susceptible to oxidative inactivation, but whole-cell catalytic activity was measured in sealed reaction vessels at 40°C, which should not significantly affect enzyme activity.

To improve D-carbamoylase thermostability or specific activity, this study introduced Cys279→Ser279 and Ile286→Ala286 point mutations [30-31] into the enzyme, but both mutations had negative effects (data not shown). The reasons for low D-carbamoylase activity require further investigation. Whether adjust-

ing promoter strength to balance expression levels and optimize the expression ratio between the two enzymes can maximize catalytic efficiency remains a topic for future research.

Strain *B. subtilis* LSL02/pUBSC possesses dual D-hydantoinase and D-carbamoylase activities and offers several advantages as a whole-cell catalyst for dual-enzyme D-HPG production: it uses common fermentation raw materials, the inducer acetoin is inexpensive, fermentation time is less than 24 h, biomass reaches 10^9 cells/mL, reaction temperature is below 45°C, substrate conversion can reach 100% without D-CpHPG intermediate accumulation, reaction duration can last 12 h, and D-HPG yield is no less than 80%. If the issue of low D-carbamoylase activity can be resolved, recombinant *B. subtilis* will be fully capable of meeting industrial D-HPG production requirements.

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