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The whole-cell immobilization of D-hydantoinase-engineered *Escherichia coli* for D-CpHPG biosynthesis

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ABSTRACT

Background: D-Hydroxyphenylglycine is considered to be an important chiral molecular building-block of antibiotic reagents such as pesticides, and β-lactam antibiotics. The process of its production is catalyzed by D-hydantoinase and D-carbamoylase in a two-step enzyme reaction. How to enhance the catalytic potential of the two enzymes is valuable for industrial application. In this investigation, an *Escherichia coli* strain genetically engineered with D-hydantoinase was immobilized by calcium alginate with certain adjuncts to evaluate the optimal condition for the biosynthesis of D-carbamoyl-p-hydroxyphenylglycine (D-CpHPG), the compound further be converted to D-hydroxyphenylglycine (D-HPG) by carbamoylase.
Results: The optimal medium to produce D-CpHPG by whole-cell immobilization was a modified Luria-Bertani (LB) added with 3.0% (W/V) alginate, 1.5% (W/V) diatomite, 0.05% (W/V) $CaCl_2$ and 1.00 mM $MnCl_2$. The optimized diameter of immobilized beads for the whole-cell biosynthesis here was 2.60 mm. The maximized production rates of D-CpHPG were up to 76%, and the immobilized beads could be reused for 12 batches.
Conclusions: This investigation not only provides an effective procedure for biological production of D-CpHPG, but gives an insight into the whole-cell immobilization technology.
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1. Introduction

The biosynthesis or enzymatic synthesis of chiral molecules, crucial for the production of most pharmaceutical drugs, has been an interesting topic in enantioselective synthesis in recent decades. The enantiopure or highly enantioenriched D-amino acids are considered to be the major chiral molecular building-blocks for a mass of biologically active compounds such as pesticides, β-lactam antibiotics and semisynthetic antibiotics, all of which were widely used to treat pathogens and share up to more than half of the world market of antibacterial drugs and peptides [1].

D-HPG is the lead compound for the production of antibiotics Amoxicillin (p-hydroxy-ampicillin), and it could be an adjunct in a pharmaceutical delivery system as it was used to improve L-dopa absorption through the intestinal peptide transporter I [2]. With enzymatic synthesis in vitro, the hydantoinase and carbamoylase are used in a two-step reaction to produce these side chain moieties [3,4]. In industrial application, there are two major industrial procedures for the production of D-HPG: batch process and continuous process. Most of the D-HPG on marker is produced by free-cells batch procedure on industrial scale. As a counterpart, the in-vivo biosynthesis of chiral molecules including D-HPG by whole-microbial-cell immobilization and its application, which has been an active concern for nearly 30 years [5,6,7,8,9,10], provides various unique advantages including relatively easier cell mass separation from the bulk liquid for possible further re-use, prevention against washout, decreasing risk of contamination and operative stability. By far, the immobilization techniques have been applied to produce proteases [11], dextran, bioethanol [12,13], L-sorbose [14] and fructooligosaccharide [15]. However, few research has studied about the biosynthesis of D-CpHPG and D-HPG with immobilized cells.

Natural alginate is a high-molecular-weight extracellular copolymer [16], consisting of uronic acid monomers β-D-mannuronate and its C-5 epimer α-L-guluronate [17,18]. It is capable to bind divalent cations and water [19]. Recently it has been applied to the immobilization of *Bacillus amyloliquens* producing antimicrobial and *Escherichia coli* producing β-galactosidase, and the corresponding production of the targets increased greatly [20,21]. With its promising characteristics, the natural alginate was designed to directly immobilize the engineered *E. coli* cells in our investigation for the bio-synthesis of D-CpHPG.

The production of D-CpHPG immobilized by calcium alginate as the supporting material was evaluated against different adjuncts including diatomite, glutaraldehyde, $CaCl_2$, and $MnCl_2$ in this investigation. The

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effect of the cell-immobilization on the fermentation time and production of D-CpHPG was compared with that of un-immobilized cells. The result promised significant improvements for future industrial application of D-CpHPG production by cell-immobilization biosynthesis.

2. Materials and methods

2.1. Genetic engineering of D-hydantoinase

The D-hydantoinase gene was separated from *Streptomyces* library saved in our laboratory. The *Streptomyces* library was screened out of a marine sediment sample gathered at a depth of ca. 40 m from Heishijiao Bay, Dalian, China in 2008. The D-hydantoinase gene was amplified by polymerase chain reaction (PCR) with a set of primers, F1 (CGCGGATCCGCGGACATCATTATCAAAAACGGAA) and F2 (CCCAAGCTTGGGTTA ATGCCGGTTTACTGCTGTATTG), which were incorporated BamHI and HindIII restriction sites (underlined), respectively. The PCR product, cleaved by BamHI and HindIII, was subcloned into plasmid pET28aα(+) to construct recombined pET28-DHase plasmid. Subsequently, the plasmid was transformed into *E. coli* BL21(DE3) to obtain the engineering bacterial. As a result, the genetically engineered *E. coli* was capable of converting DL-hydroxyphenylhydantoin (DL-HPH) into D-CpHPG. The bacterial stock was stored at -80°C for further investigations.

2.2. Culture conditions

The engineered bacteria were grown in Luria Bertani (LB) medium containing 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L deionized water. The medium pH was adjusted to 7.2 and the medium was sterilized at 121°C for 30 min [17]. The reaction of catalyzing DL-HPH to D-CpHPG was performed in 500 mL sterilized flasks with 200 mL LB and 1.9 g DL-HPH.

2.3. Determination of enzymatic properties of the D-hydantoinase

The optimal temperature was determined with sodium phosphate buffer (pH 8.0) for 10 min at 30, 40, 50, 60, 70, and 80°, respectively. To determine the pH profile of the purified D-hydantoinase in reaction, its activity was measured at 40°C in the buffers of 100 mM sodium acetate (pH 4–6), 100 mM sodium phosphate buffer (pH 6–8) and 100 mM sodium carbonate buffer (pH 9–10), respectively, with the substrate. The activity of D-hydantoinase was determined as described by previous studies [22].

As to the thermostability of D-hydantoinase, the purified enzyme was incubated, without substrate, for different times (10, 20, 30, 40 h) in 100 mM sodium phosphate buffer pH 8.0 at 40°C. The residual activity was determined based on previous studies [22]. The original activity of D-hydantoinase without pre-incubation was defined as 100%.

2.4. Preparation of immobilized cells and beads

The genetically engineered *E. coli* BL21 (DE3) carrying the pET28-DHase plasmid was inoculated in a 500 mL flask containing 200 mL LB. The culture was incubated at 37°C, 200 rpm with the addition of 50 μg/mL kanamycin. When the culture reached an OD_{600} of 0.6–0.8, IPTG was added to a final concentration of 0.1 mM, and the cells were cultivated at 16°C with shaking at 180 rpm for 12 h [23,24]. The cultures were harvested by centrifugation at 4500 g for 15 min at 4°C, and then re-suspended in reaction solution. Mixed with sodium alginate solution (3.0%, W/V), suspended cells (≥10^8 cells/mL) were passed through a syringe or peristaltic pump into $CaCl_2$ solution, which was prepared by dissolving 3 g of $CaCl_2$ powder into 100 mL deionized water to a concentration of 3.0% (W/V) [25]. The mixture was kept at 4°C for 8 h. The prepared beads were harvested from the solution, and the residual $CaCl_2$ on beads was washed away with deionized water. All steps were performed under aseptic conditions (Fig. 1).

2.5. Evaluation of immobilization parameters in batch process

The immobilization parameters including the concentrations of sodium alginate, diatomite, glutaraldehyde, $CaCl_2$ and $MnCl_2$ for the bio-synthetical conversion of DL-HPH to D-CpHPG were evaluated. Repeated batch-fermentation experiments were performed in the same bioreactor. After each batch-fermentation, the beads were washed with cold 100 mM sodium phosphate buffer (pH 8.0). The batch fermentation was repeated until the production of D-CpHPG apparently decreased. Samples were taken out from LB medium and analyzed with high pressure liquid chromatography (HPLC) every 12 h.

2.6. Measurement of the mechanical strength of beads

The mechanical strength of sodium alginate beads embedding the genetically engineered *E. coli* BL21 (DE3) was determined by using a shaking bio-reactor with appropriate amounts of beads mixture at

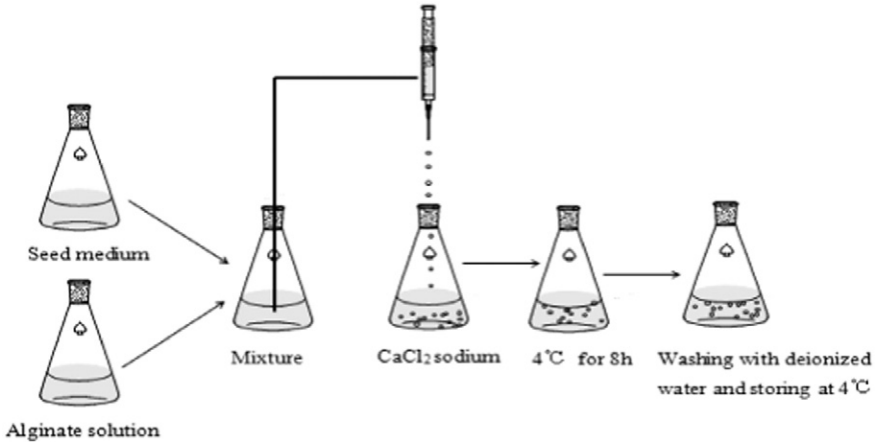

Fig. 1. Flow chart of whole cell immobilization: first, an 18 h seed culture and alginate solution were compared; second, the mixture of seed medium and alginate solution was passed through a syringe into 200 mL $CaCl_2$ (3%, W/V) solution in bacteria-free environment to produce beads; third, the $CaCl_2$ solution with beads was stored at 4°C for 8 h; fourth, the beads were washed with deionized water and stored at 4°C.

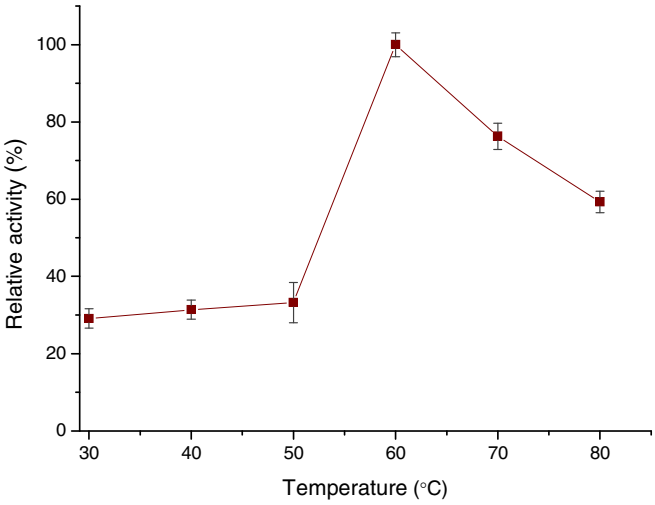

Fig. 2. Temperature optimization. The enzymatic activity of D-hydantoinase was measured in sodium phosphate buffer (pH 8.0) for 10 min at 30, 40, 50, 60, 70, and 80°C.

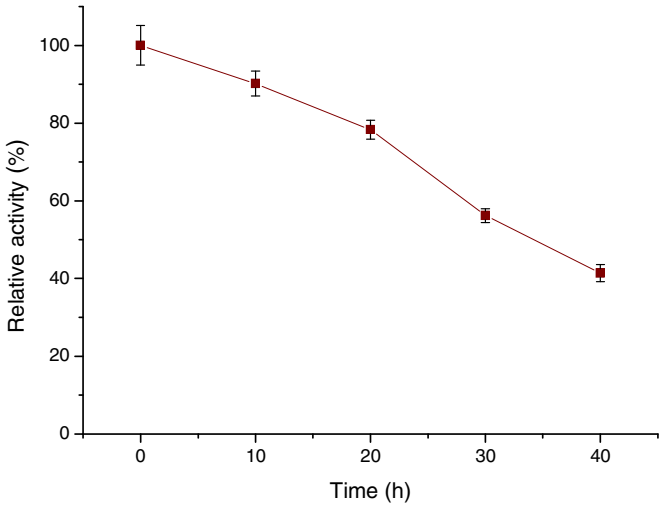

Fig. 4. Thermostability of D-hydantoinase. The purified enzyme was tested by incubation, without substrate, for different times (10, 20, 30, 40 h) in 100 mM sodium phosphate buffer pH 8.0 at 40°C. The residual activity was measured, with the original activities of D-hydantoinase, without pre-incubation, being defined as 100%.

37°C, 200 rpm for 24 h. The amounts of survived beads were counted in a standard that the values of diameter variation were within 5% compared to the fresh beads [14].

2.7. Bio-conversion analysis with HPLC

The concentration of DL-HPH and D-CpHPG produced in bio-synthetic were measured by HPLC (Agilent technologies 1200 series, USA) with a Hypersil GOLD C8 (250 mm × 4.6 mm) at a flow rate of 1 mL/min, and UV detection at 267 nm. The mobile phase was consisted of water, methanol, and trifluoroacetic acid at a ratio of 95:5:0.01 [22].

2.8. Statistical analysis

Statistical difference was analyzed by using students' t-test. The samples of significant effect resulted in a 95% ($P < 0.05$) confidence level.

3. Results

3.1. Characterization of enzymatic properties of D-hydantoinase

The optimal temperature for the reaction of D-hydantoinase was experimentally determined to be around 60°C (Fig. 2). The specific activities of the D-hydantoinase at various pHs were measured by using DL-HPH as the substrate. As shown in Fig. 3, the D-hydantoinase was active over the pH range 7.0–10.0, with the maximum at pH 8.0.

The thermal stability of the enzyme at pH 8.0 was assayed by measuring the residual activities at 40°C at different time points. The purified D-hydantoinase retained approximately 54% of its initial activity after incubation for 30 h at pH 8.0 (Fig. 4).

3.2. Effect of the concentration of calcium alginate

Immobilized cells have been widely used to improve the yield of certain pharmaceutical drugs. Alginate, with the advantages of

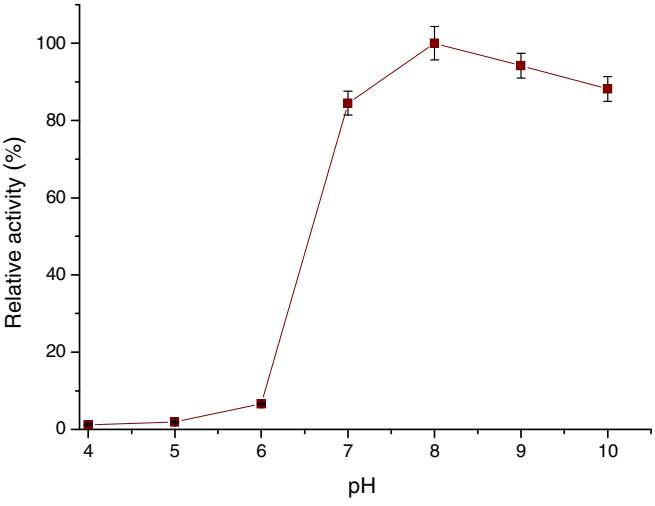

Fig. 3. Effects of pH on the specific activity of purified D-hydantoinase. The enzymatic activity was measured at 40°C using 100 mM sodium acetate buffer (pH 4–6), 100 mM sodium phosphate buffer (pH 6–8) and 100 mM sodium carbonate buffer (pH 9–10) with the substrate.

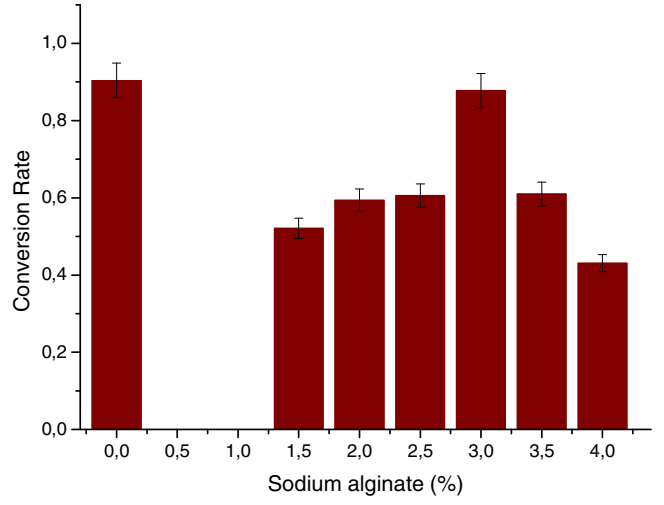

Fig. 5. Effect of concentration of calcium alginate on the production of D-CpHPG. From 0.0% to 4.0% the fluctuating concentration of sodium alginate increased by 0.5%. Reagents and conditions: LB (200 mL), DL-HPH (1.9 g), bead diameter = 2.60 mm, pH = 8.0, T = 37°C, samples were taken at 48 h.

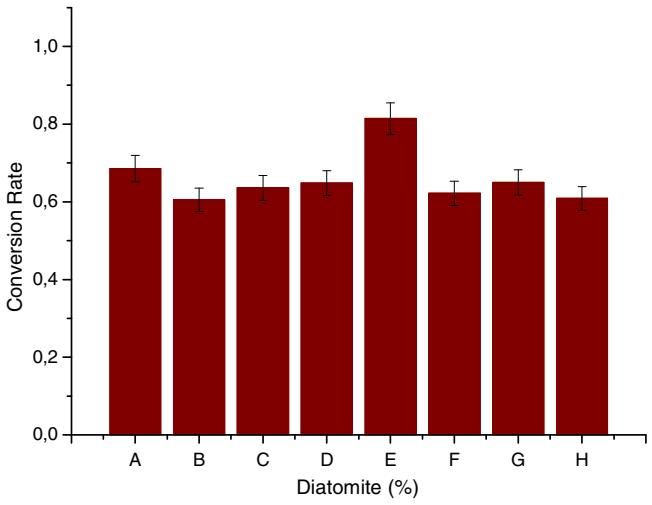

Fig. 6. Effect of concentration of diatomite on the production of D-CpHPG. From 0.0% to 3.0% the fluctuating concentration of diatomite increased by 0.5%. Reagents and conditions: LB (200 mL), DL-HPH (1.9 g), bead diameter = 2.60 mm, pH = 8.0, T = 37°C, 3.0% sodium alginate, samples were taken at 48 h. A: free cells; B: 0.0%; C: 0.5%; D: 1.0%; E: 1.5%; F: 2.0%; G: 2.5%; H: 3.0%.

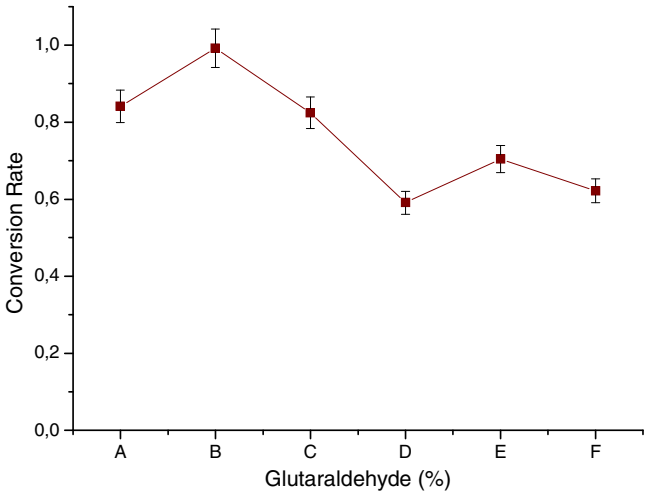

Fig. 7. Effect of concentration of glutaraldehyde on the production of D-CpHPG. From 0.0% to 1.0% the fluctuating concentration of glutaraldehyde increased by 0.25%. Reagents and conditions: LB (200 mL), DL-HPH (1.9 g), bead diameter = 2.60 mm, pH = 8.0, T = 37°C, 3.0% sodium alginate, 1.5% diatomite, samples were taken at 48 h. A: free cells; B: 0.0%; C: 0.25%; D: 0.5%; E: 0.75; F: 1.0%.

non-toxicity and inexpensiveness, was frequently used in the bio-synthetic technology. Herein, the immobilized cells embedded in alginate beads, compared to free cells, required more time to convert DL-HPH to D-CpHPG in batch process (Fig. 5). The production rate of D-CpHPG was 90% in the fermentation of free cells after 48 h, whereas in immobilized beads containing 3.0% (W/V) sodium alginate, the compound was produced at a rate of approximately 88%. The beads, prepared with higher concentration of sodium alginate, decreased the production due to stronger surface tension and lower DL-HPH diffusion rate. However, the beads, prepared with lower concentration of sodium alginate, were very fragile. Thus, most of them were broken during fermentation. As a result, 3.0% (W/V) sodium alginate showed the best balance in production and fragility, and was set for further determining the optimal diatomite concentration.

3.3. Effect of the concentration of diatomite

As an important factor in the growth and reproduction of aerobic bacterial, oxygen is indispensable, and its diffusion may be the bottleneck for the production of D-CpHPG. To improve the diffusion of oxygen, diatomite has been widely used to transfer oxygen to the immobilized beads because of its advantage of natural porous structure [14]. Different diatomite concentrations resulted in different production rates of D-CpHPG (Fig. 6). The production rate of immobilized beads containing 1.5% (W/V) diatomite was 81% at 48 h and the beads displayed higher mechanical strength (Table 1). The result indicated that diatomite could enhance the yield of D-CpHPG, and the maximum yield was achieved at the concentration of 1.5% (V/V) in our case (Fig. 6).

3.4. Effect of glutaraldehyde

Glutaraldehyde, one cross-linkers to thermally stabilize enzymes for repeatability of batch-reaction and yield enhancement, is often used in cell immobilization [23,26]. However, our study indicated that the addition of glutaraldehyde decreased the production of D-CpHPG, possibly due to the increasing viscosity, which is obstructive to the mass transfer process (Fig. 7). Thus, we did not use any glutaraldehyde in the immobilized beads.

3.5. Effect of bead diameter

Kumaravel and Gopal [20] have reported that the production of antimicrobial protein reached its maximum with the bead-diameter being 3.0 mm. But, in our research, out of approximately 90% of the cases, the D-CpHPG produced by the beads with diameter of 2.60 mm at 48 h were 20% higher than that produced by free cells (Fig. 8). The production was not good when the bead diameter was 3.03 mm. Increasing and decreasing bead-diameter beyond the value of 2.60 mm did not improve the production of D-CpHPG obviously. A bigger bead diameter resulted in lower production of D-CpHPG because of the decreased surface area to volume ratio, which is significantly crucial for the transfer process.

3.6. Effect of the concentration of $CaCl_2$ and $MnCl_2$

Metal ions were recently reported to play a significant role in the catalytic reaction of D-hydantoinase [27]. Additionally, the Mn^{2+} was previously used to enhance the activity of D-hydantoinase. Our result indicated that the optimal concentration of Mn^{2+} for D-hydantoinase's

Table 1
Various mechanical strength.

Effect factor	Concentration of alginate								Parameters		
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	Diatomite (1.5%, W/V)	$CaCl_2$ (0.05%, W/V)	$MnCl_2$ (1.0 mM)
Mechanical strength	-	-	+	+	+	++	++	++	++	+++	+++

Note: -:low mechanical strength; +:high mechanical strength; ++:higher than + mechanical strength; +++:higher than ++ mechanical strength.

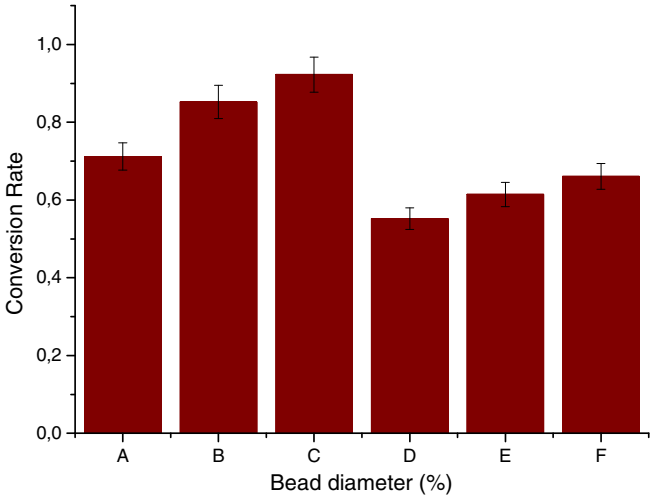

Fig. 8. Effect of bead diameter on the production of D-CpHPG. Reagents and conditions: LB (200 mL), DL-HPH (1.9 g), pH = 8.0, T = 37°C, 3.0% sodium alginate, 1.5% diatomite, samples were taken at 48 h. A: free cells; B: 2.27 mm; C: 2.60 mm; D: 3.03 mm; E: 3.54 mm; F: 4.00 mm.

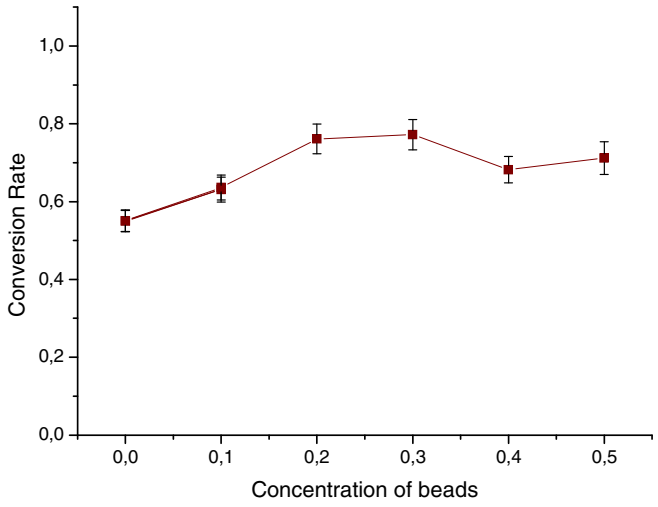

Fig. 10. Effect of bead concentration on the maximum production of D-CpHPG. From 0.0% to 50% the fluctuating concentration of beads increased by 10%. Reagents and conditions: LB (200 mL), DL-HPH (1.9 g), bead diameter = 2.60 mm, pH = 8.0, T = 37°C, 3.0% sodium alginate, 1.5% diatomite, 0.05% $CaCl_2$, 1.0 mM $MnCl_2$, samples were taken at 48 h.

function was 1.0 mM, consistent with what Chern and Chao [27] reported. The conversion rate of DL-HPH was 98% at this concentration of Mn^{2+} (Fig. 9).

The mechanical strength was highly influenced by the concentration of Ca^{2+}. Therefore, the concentration of Ca^{2+} can be used to change the mechanical strength of immobilized beads. The culture with 0.05% $CaCl_2$ added displayed highest conversion of DL-HPH (Fig. 9).

As a result, $MnCl_2$ (1.0 mM) and $CaCl_2$ (0.05%) were added into the reaction solution to improve the physical strength of beads and the production of D-CpHPG (Table 1 and Fig. 9).

3.7. Effect of concentration on the maximum production of D-CpHPG

In our study, the concentration of bead from 10% to 50% was tested for the maximum production. The beads of different concentrations were mixed in the culture with DL-HPH (9.5 g). Different bead concentration gave out different yields of D-CpHPG. The maximum production were 7.2 g (bead concentration at 30%) and 7.3 g (bead concentration at 40%) (Fig. 10). The conversion rates were 76% and 77%, respectively. To reduce cost, the 30% concentration of beads was selected as the optimum to produce D-CpHPG.

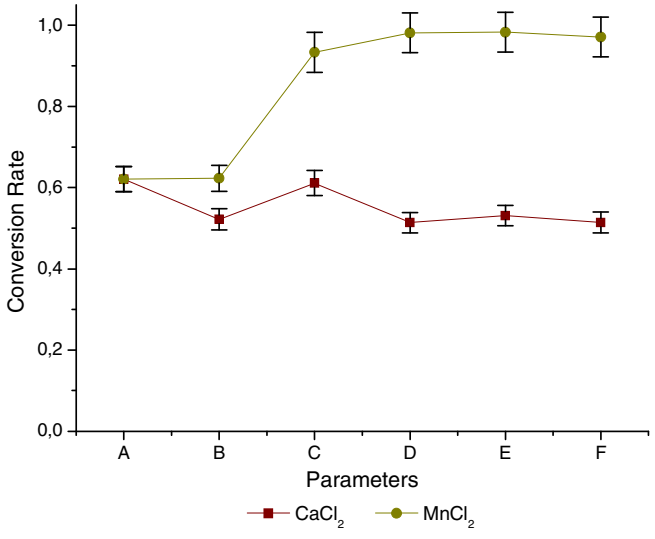

Fig. 9. Effect of the concentration of $CaCl_2$ and $MnCl_2$ on the production of D-CpHPG. The concentration of $CaCl_2$ and $MnCl_2$ were fluctuating from 0.0% to 0.2% and 0.0 mM to 2.0 mM, respectively. Reagents and conditions: LB (200 mL), DL-HPH (1.9 g), bead diameter = 2.60 mm, pH = 8.0, T = 37°C, 3.0% sodium alginate, 1.5% diatomite, samples were taken at 48 h. A: free cells; B: 0%; C: $CaCl_2$ (0.05%) and $MnCl_2$ (0.5 mM); D: $CaCl_2$ (0.1%) and $MnCl_2$ (1.0 mM); E: $CaCl_2$ (0.15%) and $MnCl_2$ (1.5 mM); F: $CaCl_2$ (0.2%) and $MnCl_2$ (2.0 mM).

3.8. Repeated batch fermentation

To evaluate the stability of the cell immobilized in the alginate, the beads were re-used for repeated reaction until the production of D-CpHPG apparently decreased. Granules containing immobilized beads were recycled for 12 consecutive batches, and they kept stable over a period of 24 d with a conversion rates of 88% (Fig. 11). However, the production of D-CpHPG sharply decreased after the 12th batch.

4. Discussion

It has been well recognized that most of protein immobilizations generally require labor-intensive pre-purification of proteins.

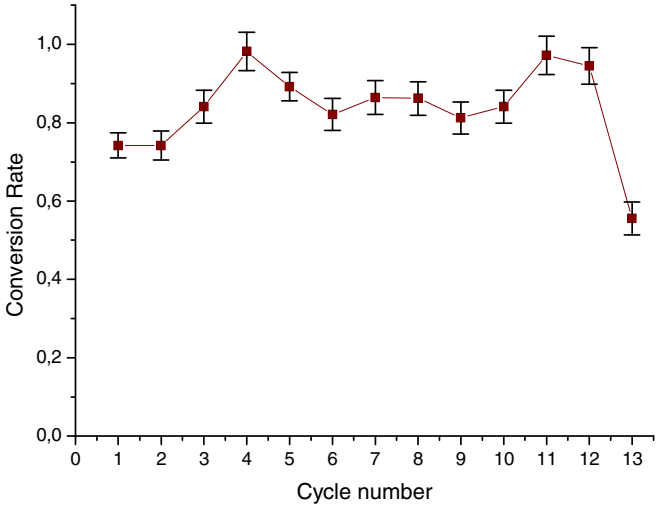

Fig. 11. Effect of repeated batch fermentation on the production of D-CpHPG. Reagents and conditions: LB (200 mL), DL-HPH (1.9 g), bead diameter = 2.60 mm, pH = 8.0, T = 37°C, 3.0% sodium alginate, 1.5% diatomite, 0.05% $CaCl_2$, 1.0 mM $MnCl_2$, samples were taken at 48 h.

Immobilization of cells would overcome this disadvantage. Cell immobilization techniques have been applied in many different industrial processes. However, few studies have been examined immobilized cell systems for D-CpHPG production. The encapsulation of living cells into a matrix that provides cells with a 3D environment has been widely studied [28]. Alginate, with the advantages of non-toxicity and inexpensiveness, was frequently used in cell-immobilization [29, 30]. A large amount of oxygen is required for the bio-production of D-CpHPG. To enhance the diffusion of oxygen in beads, diatomite is widely used for transferring oxygen into immobilized beads because of its advantage of natural porous structure. In this study, by using a combined carrier of sodium alginate and diatomite to enhance the mechanical strength and oxygen transferring performance, immobilized cells remained integrate and showed a satisfactory D-CpHPG converting efficiency.

The essential purpose of this study was to evaluate the effect of cell-immobilization against different conditions on the improvement of the production of D-CpHPG, which could be further converted into D-HPG. The appropriate mechanical strength of immobilized beads could help boost the production of D-CpHPG. The immobilization of the whole cell producing D-hydantoinase in alginate was fulfilled with parameter-optimized medium. The parameters included the concentrations of calcium alginate, diatomite, glutaraldehyde, $CaCl_2$ and $MnCl_2$, and bead diameter. The optimal immobilizing condition was determined to be with 3.0% (W/V) alginate, 1.5% (W/V) diatomite, 0.05% (W/V) $CaCl_2$, and 1.0 mM $MnCl_2$ in Luria-Bertani. Meanwhile, the concentration of experimental beads was evaluated from 10% to 50%. Noticeably, when it exceeded 30%, the production of D-CpHPG decreased, which might have resulted from the decrement of available contact surface area of the DL-HPH in the whole cell immobilization.

In summary, the optimal condition for preparing immobilized beads with endurable mechanical strength for the production of D-CpHPG was obtained with the genetically D-hydantoinase-engineered *E. coli* BL21(DE3) in our case, and the maximization of D-CpHPG yield was reached at a higher production rate of 76%, compared to the free cells. The mechanical strength of beads remained stable for enough long time for repeating the batch-processes of bio-synthesis. This investigation not only suggests that the immobilized beads of D-hydantoinase-engineered *E. coli* are of high potential for large-scale industrial production of D-CpHPG, but gives an insight into the whole-cell immobilization technology.

Conflict of interest

None.

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